AF / 1625 IFW



PTO/SB/21 (02-04)
Approved for use through 07/31/2006. OMB 0651-0031
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

# TRANSMITTAL FORM

*(to be used for all correspondence after initial filing)*

| | |
|---|---|
| Application Number | 09/919,195 |
| Filing Date | July 31, 2001 |
| First Named Inventor | Massaro |
| Art Unit | 1625 |
| Examiner Name | D. Margaret Seaman |
| Attorney Docket Number | 17293 DIV |

Total Number of Pages in This Submission

## ENCLOSURES *(Check all that apply)*

- ☐ Fee Transmittal Form
  - ☐ Fee Attached
- ☐ Amendment/Reply
  - ☐ After Final
  - ☐ Affidavits/declaration(s)
- ☐ Extension of Time Request
- ☐ Express Abandonment Request
- ☐ Information Disclosure Statement
- ☐ Certified Copy of Priority Document(s)
- ☐ Response to Missing Parts/ Incomplete Application
  - ☐ Response to Missing Parts under 37 CFR 1.52 or 1.53
- ☐ Drawing(s)
- ☐ Licensing-related Papers
- ☐ Petition
- ☐ Petition to Convert to a Provisional Application
- ☐ Power of Attorney, Revocation Change of Correspondence Address
- ☐ Terminal Disclaimer
- ☐ Request for Refund
- ☐ CD, Number of CD(s) _____
- ☐ After Allowance communication to Technology Center (TC)
- ☐ Appeal Communication to Board of Appeals and Interferences
- ☑ Appeal Communication to TC **(Appeal Notice, Brief, Reply Brief)**
- ☐ Proprietary Information
- ☐ Status Letter
- ☐ Other Enclosure(s) (please Identify below):

Amended Brief On Appeal

**Remarks**

Please apply any dificiencies or overpayments to deposit account 502362.

## SIGNATURE OF APPLICANT, ATTORNEY, OR AGENT

| | |
|---|---|
| Firm or Individual name | Gabor L. Szekeres |
| Signature | Gabor L. Szekeres |
| Date | January 30, 2006 |

## CERTIFICATE OF TRANSMISSION/MAILING

I hereby certify that this correspondence is being facsimile transmitted to the USPTO or deposited with the United States Postal Service with sufficient postage as first class mail in an envelope addressed to: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450 on the date shown below.

| | | | |
|---|---|---|---|
| Typed or printed name | Toni Whyte | | |
| Signature | Toni Whyte | Date | January 30, 2006 |

This collection of information is required by 37 CFR 1.5. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to 2 hours to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

*If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.*

17293 DIV

**PATENT**

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

| | |
|---|---|
| In re Application of | ) |
| | ) Examiner: D. Margaret Seaman |
| Massaro et al. | ) |
| | ) Art Unit: 1625 |
| Serial No.: 09/919,195 | ) |
| | ) |
| Filed: July 31, 2001 | ) |
| | ) |
| For: METHODS AND COMPOSITIONS FOR THE TREATMENT AND PREVENTION OF LUNG DISEASE | ) |

Certificate of Mailing

I hereby certify that this correspondence is being deposited on 1-30-06 with the United States Postal Service as first class mail in an envelope addressed to Commissioner for Patents, P.O.Box 1450, Alexandria VA 22313-1450 Mail Stop Appeal Brief

Toni Whyte

Toni Whyte

Date: January 30, 2006

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE =
BEFORE THE BOARD OF APPEALS

APPELLANT'S AMENDED BRIEF PURSUANT TO CONSOLIDATED PATENT RULES § 41.37

Honorable Commissioner of Patents
P.O.Box 1450
Alexandria, VA 22313-1450

In accordance with Consolidated Patent Rules 41.37 and in accordance with the requirement dated December 28, 2005 Appellant hereby submits its AMENDED BRIEF on Appeal. The fee required for filing the original Brief on Appeal has been paid. In the event a fee is required for the filing of the present Amended Brief on Appeal, the Commissioner is authorized to charge the undersigned attorney's deposit account No. 502362.

AMENDMENT of the previously timely filed Brief on Appeal was required by the Board's ORDER RETURNING UNDOCKETED APPEAL TO EXAMINER dated December 5, 2005 occasioned by the fact that the original Brief on Appeal filed on November 12, 2004 did not include an *Evidence Appendix* or a *Related Proceeding Appendix* as required by § 41.37(c)(1)(ix) and § 41.37(c)(1)(x), respectively. The present amended brief includes such appendices.

Except for the above-noted additional appendices and a modification of the identification of the real party in interest, there are no other substantive changes in the brief.

Real Party in Interest [§ 41.37(c)(1)(i)]

The real parties in interest are:

Allergan Inc. 2525 Dupont Drive, Irvine California 92612 by virtue of assignment recorded on Reel/Frame 013898/0170 in the United States Patent and Trademark Office, Georgetown University of Washington D.C. by virtue of assignment by employee inventors, and
Concurrent Pharmaceuticals Inc. 502 West Office Center Drive Fort Washington Pennsylvania 19034 by virtue of license from assignee(s).

## Related Appeals and Interferences [§ 41.37(c)(1)(ii)]

There are no related appeals nor interferences.

## Status of Claims [§ 41.37(c)(1)(iii)]

Claims 13 through 28 are pending and on appeal. Claims 29 and 30 were canceled in a preliminary amendment dated July 30, 2001. The subject matter of Claims 29 and 30 was patented in United States Patent No. 6,303,648, issued on October 16, 2001.

Claims 1 – 12 of the application were canceled during prosecution, in an amendment dated August 16, 2002.

## Status of Amendments [§ 41.37(c)(1)(iv)]

No amendment was filed after the final rejection of Claims 13 through 28, dated February 17, 2004. All amendments filed before the final rejection have been entered and comprise the record on this appeal.

## Summary of Claimed Subject Matter [§ 41.37(c)(1) (v)]

The claimed subject matter of independent Claim 13 is a method of treatment or prevention of alveolar destruction (a form of lung disease) of a

mammal by administering to the mammal a therapeutically effective amount of a compound which is an *antagonist* of retinoid receptors of the RAR β type, does not modulate RXR receptors and not specific to at least one other RAR receptor subtype (namely not specific to at least one of the RAR α and RAR γ subtypes. (see page 4 line 26 through page 5 line 24; page 11 lines 1 - 6.)

Dependent Claims 14 through 20 are more specific, and have additional inventive features (limitations) as follows:

In Claim 14 the RAR β antagonist is defined as not specific to RAR α receptors. (page 5, lines 2 – 6; page 9 line 26 through page 10 line 6; page 11 lines 7 - 18).

In Claim 15 the RAR β antagonist is defined as not specific to RAR γ receptors. (page 5, lines 6 – 10; page 9 line 26 through page 10 line 6; page 11 lines 7 - 18).

In Claim 16 the RAR β antagonist is defined as not specific to either RAR α or to RAR γ receptors. (page 5, lines 10 – 14; page 9 line 26 through page 10 line 6; page 11 lines 7 - 18).

In Claim 17 the RAR β antagonist is administered in the form of an inhalant. (page 12, lines 9 – 15; page 9 line 26 through page 10 line 6; page 11 lines 7 - 18).

In Claim 18 the inhalant contains RAR β antagonist which is not specific to RAR α receptors. (page 24, originally filed Claim 18; page 9 line 26 through page 10 line 6; page 11 lines 7 - 18).

In Claim 19 the inhalant contains RAR β antagonist which is not specific to RAR γ receptors. (page 24, originally filed Claim 19; page 9 line 26 through page 10 line 6; page 11 lines 7 - 18).

In Claim 20 the inhalant contains RAR β antagonist which is not specific to either RAR α or to RAR γ receptors. (page 24, originally filed Claim 20; page 9 line 26 through page 10 line 6; page 11 lines 7 - 18).

The claimed subject matter of independent Claim 21 is a method of increasing the gas-exhange surface area of a mammalian lung by administering to the mammal a therapeutically effective amount of a compound which is an *antagonist* of retinoid receptors of the RAR β type and has specific RAR modulating activity. (see page 4, lines 26 through page 5 line 2; page 5 line 17 – 24; page 17 line 22 through page 21 line 6; page 24 originally filed Claim 21).

Dependent Claims 22 through 28 are more specific, and have additional inventive features (limitations) as follows:

In Claim 22 the RAR β antagonist is defined as not specific to RAR α receptors. (page 5, lines 2 – 6; page 9 line 26 through page 10 line 6; page 11 lines 7 - 18).

In Claim 23 the RAR β antagonist is defined as not specific to RAR γ receptors. (page 5, lines 6 – 10; page 9 line 26 through page 10 line 6; page 11 lines 7 - 18).

In Claim 24 the RAR β antagonist is defined as not specific to either RAR α or to RAR γ receptors. (page 5, lines 10 – 14; page 9 line 26 through page 10 line 6; page 11 lines 7 - 18).

In Claim 25 the RAR β antagonist is administered in the form of an inhalant. (page 12, lines 9 – 15; page 9 line 26 through page 10 line 6).

In Claim 26 the inhalant contains RAR β antagonist which is not specific to RAR α receptors. (page 25, originally filed Claim 26; page 9 line 26 through page 10 line 6; page 11 lines 7 - 18).

In Claim 27 the inhalant contains RAR β antagonist which is not specific to RAR γ receptors. (page 25, originally filed Claim 27; page 9 line 26 through page 10 line 6; page 11 lines 7 - 18).

In Claim 28 the inhalant contains RAR β antagonist which is not specific to either RAR α or to RAR γ receptors. (page 25, originally filed Claim 20; page 9 line 26 through page 10 line 6; page 11 lines 7 - 18).

Grounds of Rejection to be Reviewed on Appeal [§ 41.37(c)(1)(vi)]

First ground: The Examiner rejected all pending claims pursuant to 35 U.S.C. § 112, first paragraph. In this rejection the Examiner did not make any distinction among the pending claims. On pages 2 – 6 of the final Office Action of February 17, 2004 the Examiner discussed an alleged "failure to comply with the written description requirement" (page 2 second numbered paragraph of the final Office Action) and thereafter separately an alleged failure to provide a "specification in such a way as to enable one

skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the invention". (page 3 third numbered paragraph of the final office Action). Applicant is of the view and urges that the requirement to provide a "written description" is the same as the requirement for an enabling disclosure. Therefore, these two allegedly separate grounds of rejection in the final Office Action are the same and can be discussed together.

In applicant's view with respect to the rejection pursuant to 35 U.S.C. § 112, first paragraph, applicant believes that all pending claims can be discussed as a group. The essence of the rejection is that the compounds used in the method are defined by the biological/pharmaceutical properties, namely that they need to be antagonists of RAR β receptors, and more specifically in the preferred embodiment that they should not be modulators of RAR α receptors, or of RAR γ receptors. The Examiner made this ground of the rejection on the basis that there is no chemical structural limitation set forth for the compounds having such biological/pharmacological activity. It is true that in the instant claims there is no structural limitation for the compounds which can be used in the method. However, as it is explained in more detail in the "arguments' below, applicant is of the view that the description of the required biological/pharmacological properties is sufficient as an enabling disclosure. Therefore the ultimate holding of insufficient disclosure is in serious error.

Second ground: All pending claims were rejected pursuant to 35 U.S.C. § 102 as being anticipated by each of the following references:

*Yu* CA 112:230122, abstract 1990;

*Song* Proc. Natl. Acad. Sci, USA Vol 91, pp 10809-10813, 1994;

*Wu* The EMBO Journal, Vol 16(7), pp 1656 – 1669, 1997;

*Xu* CA 127::16087, abstract 1997;

*Cong* American Journal of Physiology, 275(2, pt.1) L239-L246, 1998;

*Ghaffari* American Journal of Physiology, 276(3, pt.1) L398-L404, 1999.

The Examiner stated as grounds of the rejection that *Ghaffari* discloses compounds that cause modulation of RAR receptors (called "RAR modulation" in the final Office action); *Cong* discloses RAR modulation having a role in the development of the lung, and that the remaining references also teach RAR modulation in lung tissue. (see page 7 of the final Office Action of February 17, 2004). The Examiner's position appears to be (and for a holding of anticipation logically must be) that each of these references disclose within their "four corners" all elements of the claimed invention, expressly or inherently. For the reasons stated in the "argument" below, this position is in serious error.

In applicant's view, with respect to the rejection pursuant to 35 U.S.C. § 102 the claims should be divided in at six separate groups of inventiveness and the erroneous nature of this rejection merits discussion with respect to each group.

Argument [§ 41.37(c)(1)(vii)]

Rejection Pursuant to 35 U.S.C. § 112, first paragraph; All Pending Claims Discussed as a Group

The Examiner asserts that the disclosure is insufficient and not enabling to a person of ordinary skill in the art "because the scope of the claims is unknown due to the *structure limitations* not being specifically disclosed." (page 2 of the final Office Action of February 17, 2004, italics added). This assertion is in serious error because there is no requirement in patent law that the chemical structure of a claimed group of compounds must be disclosed. It is well established law that a person of ordinary skill in the art *can be enabled* by describing the physical or biological properties of a class of compounds which are used in a claimed

method. As it will become apparent below, this requirement is satisfied in connection with the instant claims.

The Examiner stated that,

> "the claimed invention invention is drawn to compositions that have RARβ antagonist having specific RAR modulating activity and a method of of treating using such compositions. However, the only compounds that are enabled by the instant specification have already been patented." (Page 3 of the final Office Action)

The just quoted statement from the final Office Action that rejects the claims pursuant to 35 U.S.C. § 112, first paragraph is indicative that the

Examiner has confused the requirements for patenting new compositions and patenting methods using old compositions. The law is well established that a new and unobvious use of an old composition is patentable. The statement is grossly in error because the instant claims are not drawn to compositions. They are drawn only to methods of treatment.

The Examiner also stated that,

> "The only compounds that fit within the bounds of the instant claim 13 are the compounds/methods of the US Patent #6,303,648. The instant specification does not have written description as to how to make compounds that fit within the instant parameters outside the compounds of the parent patent." (page 3 of the final Office Action)

The just quoted statement in the final Office Action also reveals a serious misconception or misreading of the instant disclosure. On page 12 line 30 through page 13 line 8 the instant specification teaches that the "synthesis of candidate compounds having specific RAR modulating activity is well known in the art". The term "*specific RAR modulating activity*" is defined on page 5 lines 17 – 24. The essence of this definition is that compounds having "specific RAR modulating activity" bind <u>at least</u> ten times better to RAR receptors than to RXR receptors. Page 5 line 25 through page 6 line 6 briefly describe the assay or method by which the "specific RAR modulating activity" of a compound can be *routinely* determined, and incorporates by reference the disclosures (U. S. Patent No.

5,776,699 and PCT Publication No. W093/11755) where the nature of the required assays is described in precise detail. Still further, on page 13 line 9 through page 14 line 20 the specification again describes, and in more detail, routine assays for determining "specific RAR modulating activity" and routine assays for measuring the dissociation constant (Kd) of a given ligand with retinoid receptors. This part of the specification again refers to and incorporates by reference United States Patent Nos. 5,455,265 and 5,776,699.

Moreover, the passage on Page 12 line 30 through page 13 line 8 of the instant specification incorporates by reference U.S. Patent Nos. 5,739,338; 5,728,846; 5,760,276 and 5,877,207 each of which describes "the synthesis of RAR ligands having antagonist and/or inverse agonist activity. These patents have general formulas of broad scope and also list numerous exemplary compounds of specific disclosed structure.

Therefore, the statement in the final Office Action that "The only compounds that fit within the bounds of the instant claim 13 are the compounds/methods of the US Patent #6,303,648", while technically may be correct, is a misstatement in context, because with the numerous structures of broad scope and the numerous specific examples of the patents incorporated by reference the instant specification teaches a large number of compounds usable in the present invention, or suitable for undergoing the routine assays for "specific RAR modulating activity" and for specific or selective RARβ antagonist activity.

The Examiner refers to the factors of *in re Wands* 858 F.2d 731, 737, 8 USPQ2d 1400, 1404 (fed. Cir. 1988). Applicant urges that the factors of enablement in *in re Wands* "are illustrative, not mandatory. What is relevant depends on the facts." *Enzo Biochem Inc. v. Calgene Inc.,* 52 USPQ2d 1129 (Fed. Cir. 1999).

Nevertheless, even applying the *Wands* factors the holding of a lack of enablement is in serious error. Specifically, the claims are broad but the nature of the invention is commensurately broad in that the applicant discovered that antagonists of RARβ retinoid receptors which do not have significant RXR modulating activity, and preferably have no RARα nor RARγ modulating activity, are suitable to be used for curing or preventing certain lung diseases or deficiencies in mammals. The state of the prior art and the level of skill in the art are such, as evidenced by the patents incorporated by reference, that a person of ordinary skill can routinely screen compounds for their above-noted biological/physiological profile. In the specification Applicant provides clear and routine methods of screening as directions for determining the suitability of any compound for use in the method of the present invention. The specification provides one exemplary compound of specific structure as a working example but the compounds incorporated by reference provide a multitude of additional working examples. Finally, the experimentation needed for determining the suitability of a compound to be used in the methods of the invention is mere routine experimentation easily performed by persons who have ordinary but nevertheless high skill in the art. Such routine testing or assaying should

not be considered undue experimentation especially in view of the fact that the prior art includes hundreds of patents describing compounds which have retinoid receptor modulating activity and are therefore candidates to be screened for the required "specific RAR modulating" and RARβ antagonist properties.

In a previous Office Action the Examiner stated that there is no direction in the specification for a person of ordinary skill in the art what compounds to screen for the required biological/pharmacological activity of the compounds used in the instant invention. Therefore, in the Examiner's view, aspirin is as likely to be tested as any other drug. Page 5 of the Office Action of September 15, 2003) This observation, or ground of the rejection is in serious error for the following reasons. A multitude of compounds are described and/or known in the pharmaceutical arts to be modulators of retinoid receptors (retinoids). Persons of ordinary skill in the art tend to be scientists with advanced degrees who are likely to be aware of the "retinoid receptor modulating" nature of a compound and are likely to screen only such compounds. They are highly unlikely to screen aspirin.

In the final Office Action the Examiner states the additional reason for the "lack of enablement" rejection,

> "Furthermore, no information is presented as to how the undisclosed antagonist compound would have been administered to treat an unspecified disease. Thus, the skilled artisan would not have been able to practice the steps required by the claimed invention." (Page 6 of the final Office Action)

The just quoted passage in the final Office Action is in error. On page 11 line 21 through page 12 line 29 the specification teaches that medical conditions such as emphysema and BPD (brochopulmonary dysplasia) can be treated in accordance with the present invention. This section of the specification also describes modes of administration, for example as an inhalant, or a composition suitable for systemic administration (intravenous and/or intraperitonial). Pharmacological vehicles for all these methods of administration are well known in the art. (see page 12 lines 16 – 29).

In view of the novel and unobvious discovery that compounds having "specific RAR modulating" and RARβ antagonist properties are useful for treating or preventing certain lung disorders, the multitude of prior art "retinoid compounds" provide a multitude of exemplary compounds to be subjected to the routine assays described in the instant specification to obtain a multitude of compounds suitable to be used in the novel method of the invention.

In light of the foregoing the rejection of all pending claims for a lack of adequate written description and/or lack of enabling disclosure is in serious error and should be reversed.

Rejection Pursuant to 35 U.S.C. § 102; Claims Discussed in Six Separate Groups

Comments Applicable to All Claims:

The grounds provided in the final Office Action and in the previous Office Actions for rejecting the claims as "anticipated" by each of the cited references is insufficiently explained, and is in serious error.

In response to the Section 102 rejection in a preceding Office Action based on these references, applicant requested the Examiner to explain:

> "If the Examiner has found an anticipatory disclosure of each and every limitation of any of the present claims in one or more of these references, Applicants respectfully request the Examiner to expressly point to such disclosure." (Reply and Amendment of August 14, 2003, page 6).

The law is well settled that an allegedly anticipatory disclosure must disclose each and every limitation of the allegedly anticipated patent claim. Applicant urges that none of these allegedly "anticipatory" references satisfy the legal requirement for anticipation and that the Examiner has never responded adequately to the just quoted request by the applicant.

Claim Group 1, Claims 13 – 16

As noted above these claims require in the method of treatment or prevention the use of a compound that is an RARβ antagonist, and not a modulator of RXR receptors. (see the definition of "specific RAR modulating activity" on page 5 lines 17 – 24 of the specification.) nor a modulator of either RARα or RARγ receptors. None of the cited references disclose all these elements or limitations. The Examiner asserts that these elements must be inherent in the compounds used in the references. For the reason explained below the assertion of inherency is in serious error.

There is a significant difference between just being a "retinoid", namely a compound having some modulating activity on any or all retinoid

receptors, and being selective to RAR receptors (not active on RXR receptors) and then being further selective by acting as an *antagonist* of RARβ and being inactive on either RARα or RARγ receptors. These distinctions or limitations of the instant claims are not expressly found in the references.

On page 2 lines 26 through page 3 line 29 the instant specification points out the well known fact that all *trans* retinoic acid (ATRA) has beneficial effect on mammals having destroyed or insufficient alveoli, by reversing the destruction and/or promoting formation of more alveoli. ATRA is a well known agonist, not an antagonist of all RAR receptors. Moreover,

> "the retinoid receptors, when bound by an appropriate ligand, are mediators of various life processes, including reproduction, metabolism, differentiation, hematopoiesis and embryogenesis." (page 3 lines 17 – 21)

Therefore, there is room, in fact a need, in the art for drugs, such as the ones used in the instant method, which tend to be more selective, treat lung diseases by promoting alveoli formation without a high likelihood of serious side effects.

It should also be noted that because ATRA is an agonist of all three RAR receptors it is a quite different and a surprising discovery of the present invention that a RARβ antagonist also has a like effect

on mammalian lungs without a high likelihood of the serious side effects that administration of ATRA may cause.

Claim Group 2, Claims 17 – 20

In addition to the features and elements discussed above, these claims require that the RARβ antagonist compound should be administered as an *inhalant*. The comments made above with respect to Claims 13 – 16 are fully applicable here, with the further comment that no cited reference includes *all the limitations*, including the call for an "inhalant" of these claims.

As with respect to all claims it is emphasized again that being a "retinoid' or a modulator of "retinoid receptors" is not the same as being an antagonist of RARβ and having no modulating activity on RXR receptors.

Claim Group 3, Claim 21

Claim 21 requires the use of an RARβ *antagonist* which is not a modulator of RXR receptors (see the definition of "specific RAR modulating activity" on page 5 lines 17 – 24 of the specification) to increase the gas-exchange surface area of a mammalian lung. Although this claim does not require the antagonist to be inactive with regard to RARα or RARγ receptors, for the reasons explained above the combination of the features or limitations recited in the claim are not present in any of the cited references, neither expressly nor inherently.

As with respect to all claims it is emphasized again that being a "retinoid' or a modulator of "retinoid receptors" is not the same as being an antagonist of RARβ and having no modulating activity on RXR receptors.

Claim Group 4, Claims 22 – 24

In addition to all features or limitations in Claim 21, these claims also have the requirement that the compound used in the treatment to increase gas-exchange surface area in the mammalian lung should be inactive as a modulator of either the RARα or RARγ receptors. For the reasons explained above the combination of the features or limitations recited in this claim are not present in any of the cited references, neither expressly nor inherently.

As with respect to all claims it is emphasized again that being a "retinoid' or a modulator of "retinoid receptors" is not the same as being an antagonist of RARβ and having no modulating activity on RXR receptors.

Claim Group 5, Claim 25

Claim 25 requires the use of an RARβ *antagonist* which is not a modulator of RXR receptors (see the definition of "specific RAR modulating activity on page 5 lines 17 – 24 of the specification) in an *inhalant* to increase the gas-exchange surface area of a mammalian lung.

Although this claim does not require the antagonist to be inactive with regard to RARα or RARγ receptors, for the reasons explained above the combination of the features or limitations recited in the claim are not present in any of the cited references, neither expressly nor inherently.

As with respect to all claims it is emphasized again that being a "retinoid' or a modulator of "retinoid receptors" is not the same as being an antagonist of RARβ and having no modulating activity on RXR receptors.

Claim Group 6, Claims 22 – 24

In addition to all features or limitations in Claim 25 that calls for an *inhalant*, these claims also have the requirement that the compound used in the treatment to increase gas-exchange surface area in the mammalian lung should be inactive as a modulator of either the RARα or RARγ receptors. For the reasons explained above the combination of the features or limitations recited in this claim are not present in any of the cited references, neither expressly nor inherently.

As with respect to all claims it is emphasized again that being a "retinoid' or a modulator of "retinoid receptors" is not the same as being an antagonist of RARβ and having no modulating activity on RXR receptors.

In light of all of the foregoing the rejection of Claims 13 –28 of the above-identified application is in error and should be reversed.

Claims Appendix [§ 41.37(c)(1)(viii)] A Claims Appendix is enclosed.

Evidence Appendix [§ 41.37(c)(1)(ix)] An Evidence Appendix is enclosed. It is noted that the evidence appendix contains only the identification and copies of the references cited by the examiner in her reliance of rejecting the claims. There is no other pertinent evidence on record.

Related Proceeding Appendix [§ 41.37(c)(1)(x)] A Related Proceeding Appendix is enclosed. It is noted that there is no related proceeding to be listed as required by § 41.37(c)(1)(ii)

Respectfully submitted

Date: January 27, 2006

Gabor L. Szekeres

Gabor L. Szekeres
Registration No. 28,675

Law Offices of Gabor L. Szekeres
8141 E. Kaiser Boulevard, Suite 112
Anaheim, CA 92808
(714) 998-3295
(714) 998-3296 fax

APPENDIX I

CLAIMS ON APPEAL pursuant to § 41.37(c)(1)(viii)

13. A method for the treatment or prevention of alveolar destruction in a mammal comprising the step of administering a therapeutically effective amount of an RARβ antagonist having specific RAR modulating activity to said mammal, and such antagonist is not specific to at least one other RAR receptor subtype.

14. The method of claim 13, wherein said RARβ antagonist is not specific to RARα.

15. The method of claim 13 wherein said RARβ antagonists is not specific to RARγ.

16. The method of claim 13 wherein said RARβ antagonist is not specific to RARα or RARγ.

17. The method of claim 13 wherein said composition is administered in the form of an inhalant.

18. The method of claim 17 wherein said RARβ antagonist is not specific to RARα.

19. The method of claim 17 wherein said RARβ antagonist is not specific to RARγ.

20. The method of claim 17 wherein said RARβ antagonist is not specific to RARα or RARγ.

21. A method to increase the gas-exchange surface area of a mammalian lung in a mammal in need thereof comprising the step of administering a therapeutically effective amount of an RARβ antagonist having specific RAR modulating activity to said mammal.

22. The method of claim 21, wherein said RARβ antagonist is not specific to RARα.

23. The method of claim 21 wherein said RARβ antagonist is not specific to RARγ.

24. The method of claim 21 wherein said RARβ antagonist is not specific to RARα or RARγ.

25. The method of claim 21 wherein said composition is administered in the form of an inhalant.

26. The method of claim 25, wherein said RARβ antagonist is not specific to RARα.

27. The method of claim 25 wherein said RARβ antagonist is not specific to RARγ.

28. The method of claim 25 wherein said RAR β antagonist is not specific to RARα or RARγ.

## APPENDIX II

EVIDENCE APPENDIX pursuant to § 41.37(c)(1)(ix)

References relied on by the Examiner to support the rejection, copies enclosed.

*Yu* Respiration Physiology, Vol 79, 101-110, 1990 {CA 112:230122, abstract 1990];

*Song* Proc. Natl. Acad. Sci, USA Vol 91, pp 10809-10813, 1994;

*Wu* The EMBO Journal, Vol 16(7), pp 1656 – 1669, 1997;

*Xu, Xiao—Chun*, Journal of the National Cancer Institute, vol. 89(9), 624-629, 1997 [CA 127::16087, abstract 1997;]

*Cong* American Journal of Physiology, 275(2, pt.1) L239-L246, 1998; *Ghaffari* American Journal of Physiology, 276(3, pt.1) L398-L404, 1999.

RELATED PROCEEDING APPENDIX pursuant to § 41.37(c)(1)(x)

There are no related proceeding.

*Respiration Physiology*, 79 (1990) 101–110
Elsevier

RESP 01624



# Indirect effects of histamine on pulmonary rapidly adapting receptors in cats

Jun Yu and Andrew M. Roberts

*Department of Physiology and Biophysics, School of Medicine, University of Louisville, Louisville, KY 40292, U.S.A.*

(Accepted for publication 2 November 1989)

**Abstract.** We did experiments to determine the relative importance of lung mechanical changes during histamine induced activation of pulmonary rapidly adapting receptors (RARs). In anesthetized, open-chest, artificially ventilated cats, we recorded RAR activity and injected histamine (25–50 μg/kg) into the right atrium. Histamine initially increased RAR activity from 1.1 ± 0.2 to 3.6 ± 0.6 imp/sec (n = 30) at 15.6 ± 0.8 sec when dynamic lung compliance ($C_{DYN}$) was decreased by 29.1 ± 1.5%. The firing pattern of RARs changed from a relatively irregular pattern to a pronounced respiratory modulation. RAR activity reached its peak (5.6 ± 0.8 imp/sec) at 36.3 ± 3.3 sec. The firing pattern further changed to a cardiac modulation, and the activity closely correlated with cardiac output. Comparing the initial response of RARs to histamine with the response to mechanically decreasing $C_{DYN}$, we found that the activities were similar when $C_{DYN}$ was decreased by the same amount. Our experiments suggest that in cats the initial increase of RAR activity in response to histamine is related to lung mechanical changes, but the later increase is related to cardiovascular functions.

Rapidly adapting receptors; Histamine; Lung compliance; Cardiac output

Pulmonary rapidly adapting receptors (RARs) are stimulated by a variety of chemicals. The response of RARs to histamine has attracted a lot of attention, since histamine is released in the airways in asthma and pulmonary anaphylaxis. Histamine, either injected intravenously or given as an aerosol, stimulates RARs in guinea pigs (Bergren and Sampson, 1982), rabbits (Mills *et al.*, 1969; Sellick and Widdicombe, 1971), cats (Armstrong and Luck, 1974) and dogs (Sampson and Vidruk, 1975; Coleridge *et al.*, 1978). The mechanism of stimulation of RAR by histamine is still uncertain and there is evidence that histamine can stimulate the receptors by both indirect mechanical effects and direct chemical effects (Pack, 1981; Sant'Ambrogio, 1987).

In rabbits (Mills *et al.*, 1969) for example, a β-adrenergic agonist which relaxes airway smooth muscle can block the RAR response to histamine as the lung mechanical

*Correspondence address:* A.M. Roberts, Department of Physiology and Biophysics, School of Medicine (Room 1115), Health Sciences Center, University of Louisville, Louisville, KY 40292, U.S.A.

changes are prevented. From this experiment it seems that lung mechanical change is the primary factor causing the stimulation. On the other hand, however, histamine causes a greater increase in RAR activity than a dose of acetylcholine which causes an equal change in lung mechanics (Vidruk *et al.*, 1977; Dixon *et al.*, 1979). Furthermore, topical application of histamine stimulates RARs without significantly influencing lung mechanics (Vidruk *et al.*, 1977). In dogs (Sampson and Vidruk, 1978), isoproterenol did not abolish the RAR response to histamine completely, although isoproterenol did reduce the tracheal pressure swings produced by histamine. These experiments suggest that histamine may have some direct chemical effects on RARs.

In previous studies (Mills *et al.*, 1969; Armstrong and Luck, 1974; Sampson and Vidruk, 1975), there has been little attempt to correlate the time course of changes in RAR activity with lung mechanical changes caused by histamine. We have not found any account of the time of onset of RAR activity in response to histamine injection into the right atrium, nor of the time taken to produce a maximal increase in firing. Therefore, in the present study, we (1) examined the time course of the RAR response to right atrial injection of histamine, and (2) tried to determine the extent that the increase in RAR activity is due to the indirect mechanical stimulation from a decrease in lung compliance.

## Methods

Experiments were conducted on 17 adult cats (2.1–5.5 kg) of either sex. Cats were anesthetized with sodium pentobarbital (30–35 mg/kg; i.v.). Supplemental doses were given by intravenous drip of 0.1% sodium pentobarbital in 0.9% saline at the rate of about 4 mg/kg/h throughout the experiments to maintain anesthesia.

*Procedures and recordings.* The trachea was cannulated low in the neck, the chest was widely open and the lungs were ventilated with 50% $O_2$ in air by a Harvard respirator. Tidal volume was set at about 8–10 ml/kg body weight. Tidal $CO_2$ was monitored by a gas analyzer. Ventilatory frequency (about 15–20 cycles/min) was adjusted to maintain end-tidal $CO_2$ at about 30–35 mm Hg. A positive end-expiratory pressure (PEEP) was maintained by placing the expiratory outlet tube of the respirator under 3–4 cm of water. Tracheal pressure was measured from a side arm of the tracheal cannula by a pressure transducer (Statham P23). The left femoral artery was cannulated to record arterial blood pressure through a Statham P23 pressure transducer. Heart rate was counted by a rate meter triggered from the pulse wave of the arterial blood pressure. In some experiments cardiac output was also measured by an ultrasonic flow probe (Transonic Systems Inc.) which was placed on the ascending aorta. These variables were recorded on a Grass polygraph. The right or left vagus nerve was dissected from the carotid sheath for recording of afferent impulses. The integrated RAR activity and other selected variables were recorded by a Grass model 7 polygraph and sometimes these variables together with RAR action potentials were recorded by a Gould (ES 1000) electrostatic recorder. Arterial blood samples were taken from a femoral artery

and their pH, $P_{CO_2}$, $P_{O_2}$
Metabolic acidosis was c
venously.

We dissected fine slips
nerve intact, and recorde
airways. The receptors
discharge and by their ra
and Larrabee, 1946; Mill
by ratemeters whose win
amplitude. Receptors w
airways.

*Measurement of $C_{DYN}$ an*
to the change in trachea
defined as the inverse c
periodically removing an
resulted in stepwise incr
*et al.*, 1986; Yu *et al.*, 19
the lungs for 3 $V_T$ abov

*Protocol.* The time cou
phosphate (50 or 100 $\mu$g
was measured over 7 ve

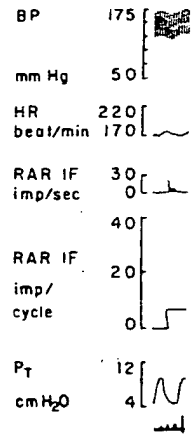



Fig. 1. Stimulation of a rapid
signal mark). BP, blood press
RAR activity at peak lung sti
cycles). Maximal RAR activ

and their pH, $P_{CO_2}$, $P_{O_2}$ and $[HCO_3^-]$ were measured by a blood gas/pH analyzer. Metabolic acidosis was corrected by administration of 2.8% $NaHCO_3$ solution intravenously.

We dissected fine slips of the right or left cervical vagus nerve, leaving the rest of the nerve intact, and recorded afferent impulses arising from RARs in the intrapulmonary airways. The receptors were identified by their characteristically irregular pattern of discharge and by their rapidly adapting response to lung inflation (2 or 3 VT) (Knowlton and Larrabee, 1946; Mills *et al.*, 1969; Yu *et al.*, 1987). Afferent impulses were counted by ratemeters whose window discriminators were set to accept potentials of a particular amplitude. Receptors were located by gently probing the lungs and intrapulmonary airways.

*Measurement of $C_{DYN}$ and lung stiffness.* $C_{DYN}$ was calculated as the ratio of tidal volume to the change in tracheal pressure during inflation by the ventilator. Lung stiffness is defined as the inverse of $C_{DYN}$. In some experiments we changed lung stiffness by periodically removing and then replacing PEEP for 2, 5, and 10 ventilator cycles, which resulted in stepwise increases in lung stiffness or stepwise decreases in $C_{DYN}$ (Jonzon *et al.*, 1986; Yu *et al.*, 1987). Then we restored lung stiffness to control by hyperinflating the lungs for 3 VT above FRC.

*Protocol.* The time course of the RAR response to a bolus injection of histamine acid phosphate (50 or 100 μg) into the right atrium was observed. RAR activity at control was measured over 7 ventilator cycles and expressed as imp/sec. RAR activity after

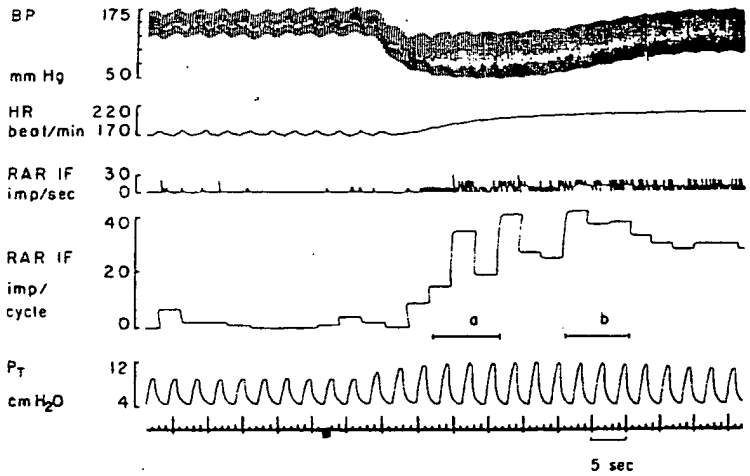



Fig. 1. Stimulation of a rapidly adapting receptor (RAR) by right atrial injection of histamine (50 μg/kg, at signal mark). BP, blood pressure; HR, heart rate; RAR IF, RAR impulse frequency; PT, tracheal pressure. RAR activity at peak lung stiffness was sampled during the period denoted by 'a' (3 consecutive ventilator cycles). Maximal RAR activity was sampled during the period denoted by 'b' (3 consecutive ventilator cycles).

histamine was calculated by counting impulses over three consecutive ventilator cycles and expressed as imp/sec. After histamine was injected, RAR activity was sampled, (1) during the maximal increase in lung stiffness (decrease in $C_{DYN}$), and (2) during the maximal activity of RARs. Figure 1 illustrates the procedure. The latencies to these two events was noted and the activities were compared.

In 13 RARs from 7 cats, the response of RARs to histamine injection was compared with that to an equivalent increase in lung stiffness. The activity was first recorded at different levels of lung stiffness, and a linear regression line was constructed, so that the activity of that particular RAR could be predicted from the regression line at an anticipated change in lung stiffness. The activities sampled at the maximal increase in lung stiffness and at the maximal activity were compared and were also compared with the RAR activity calculated from the regression line.

To determine the histamine effects on cardiovascular variables, we recorded arterial blood pressure, heart rate and cardiac output. Since cardiac output varied from animal to animal, we normalized the values of these variables to a percentage change from control.

Data reported are means ± S.E. Differences between groups were assessed by a paired Student *t*-test. The difference was considered as significant at a $P < 0.05$ level.

## Results

We recorded the impulse activity of 30 rapidly adapting receptors from 17 cats. As previously reported, during control most receptors had an irregular discharge and often had respiratory and/or cardiac modulations. RAR activity during control was low and averaged 1.1 ± 0.2 imp/sec.

*Effects of histamine on lung mechanics.* Right atrial injection of histamine changed lung mechanics. It increased tracheal pressure swing (the change in tracheal pressure). At control the change in tracheal pressure per ventilator cycle was 8.1 ± 0.5 cm $H_2O$ (n = 30). The change in tracheal pressure began to increase about 3 seconds after injection (*e.g.* the increase of pressure occurred within one or two ventilator cycles after injection). The increase of change in tracheal pressure reached its peak (11.3 ± 0.7 cm $H_2O$) at 15.6 ± 0.8 sec after injection. Calculated in terms of $C_{DYN}$, this increase of change in tracheal pressure gives a 29.1 ± 1.5% decrease in $C_{DYN}$.

*Effects of histamine on RAR activity.* RARs were stimulated by right atrial injection of histamine. RAR activity usually did not increase until 6 seconds after injection (only 3 of the 30 receptors were obviously activated within 3 seconds). RAR activity increased as lung stiffness increased. When lung stiffness increased to its peak, RAR activity increased significantly to 3.6 ± 0.6 imp/sec ($P < 0.01$). As lung stiffness increased to its peak, about half of the receptors had a clear respiratory modulation. After lung stiffness had peaked, RAR activity continued to increase, but the time course of this increase

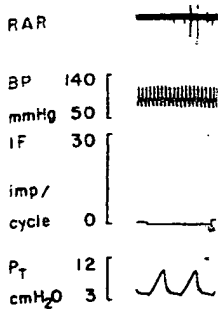



Fig. 2. Effect of right atria
RAR, RAR action potenti
cycle; $P_T$, tracheal pressu
a respiratory modulation,
Also note the response of

varied significantly. F
seconds with an aver
increased compared
peak. Although the r
clearer cardiac modu
modulation was supe
histamine injection is

*Comparison of RAR a*
13 RARs (from 7 ca
decreasing $C_{DYN}$) w
atrium. Each RAR sh
(fig. 3). The stiffer th
increased, the firing p
regular discharge with
receptors at the ma
compared the activity
increased the activity
decreased by 34.8%.
caused by mechanica
histamine injection, I
mately 15 sec after tr
7.2 ± 1.5 imp/sec, wh
of $C_{DYN}$ ($P < 0.05$).

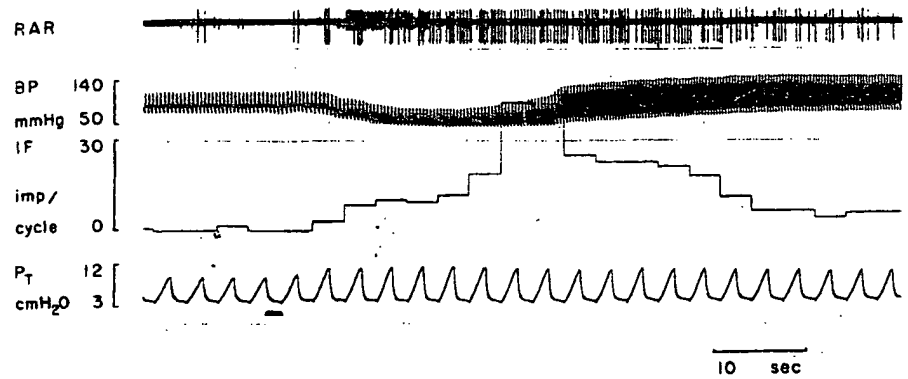



Fig. 2. Effect of right atrial injection of histamine (50 μg/kg) on rapidly adapting receptor (RAR) activity. RAR, RAR action potentials; BP, blood pressure; IF, ratemeter counts of RAR activity in each ventilator cycle; $P_T$, tracheal pressure and injection marker (black bar). Note RAR activity increased initially with a respiratory modulation, then with a prominent cardiac modulation superimposed on the respiratory one. Also note the response of a bronchial C-fiber (small spikes) at longer latency with a small burst period.

varied significantly. RAR activity reached its peak (5.6 ± 0.3 imp/sec) from 13 to 75 seconds with an average of 36.3 ± 3.3 sec after injection. The activity was significantly increased compared with the activity when the increase in lung stiffness reached its peak. Although the respiratory modulation continued, the activity began to show a clearer cardiac modulation in about three fourths of the RARs. Usually, the cardiac modulation was superimposed on the respiratory modulation. The RAR response to histamine injection is illustrated in fig. 2.

*Comparison of RAR activities evoked by decreasing $C_{DYN}$ and histamine.* We examined 13 RARs (from 7 cats) and compared their responses to increasing lung stiffness (or decreasing $C_{DYN}$) with the responses to a bolus injection of histamine into the right atrium. Each RAR showed a positive correlation between its activity and lung stiffness (fig. 3). The stiffer the lungs were, the more activity the RAR had. As lung stiffness increased, the firing pattern of RARs changed from an irregular discharge to a relatively regular discharge with a respiratory modulation. We also calculated the activity of these receptors at the maximal increase in lung stiffness after histamine injection and compared the activity with the activity evoked by increasing lung stiffness. Histamine increased the activity of 13 RARs from 1.3 ± 0.5 to 5.2 ± 1.4 imp/sec when $C_{DYN}$ was decreased by 34.8%. This increase in activity was similar to that (5.2 ± 1.2 imp/sec) caused by mechanically decreasing $C_{DYN}$ to the same amount (fig. 3). However, after histamine injection, RAR activity continued to increase and reached its peak approximately 15 sec after tracheal pressure reached a maximum. At this time the activity was 7.2 ± 1.5 imp/sec, which was significantly different from the activity at the peak change of $C_{DYN}$ ($P < 0.05$).

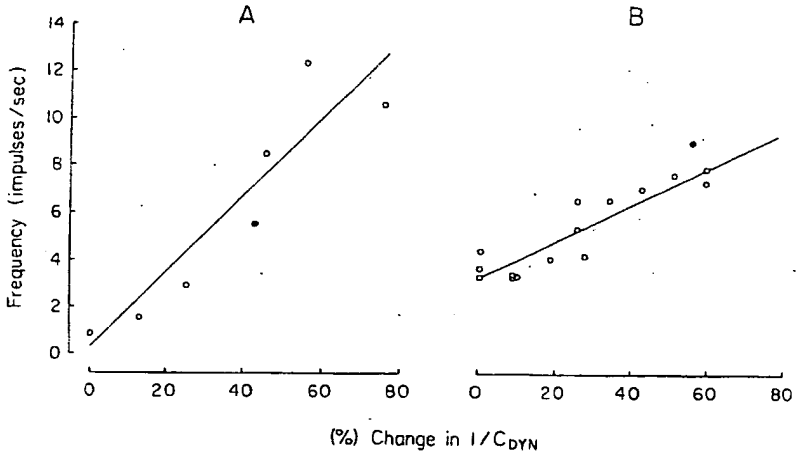



Fig. 3. Responses of two rapidly adapting receptors (RARs) to an increase in stiffness ($1/C_{DYN}$) produced by briefly removing PEEP (o) or by injecting histamine (50 μg/kg) into the right atrium (•). The data points after histamine were calculated over the three consecutive ventilatory cycles with the largest increases in lung stiffness. Linear regression lines were calculated for the open circles data. Note that RAR activity induced by histamine was comparable with that induced by increasing $1/C_{DYN}$.

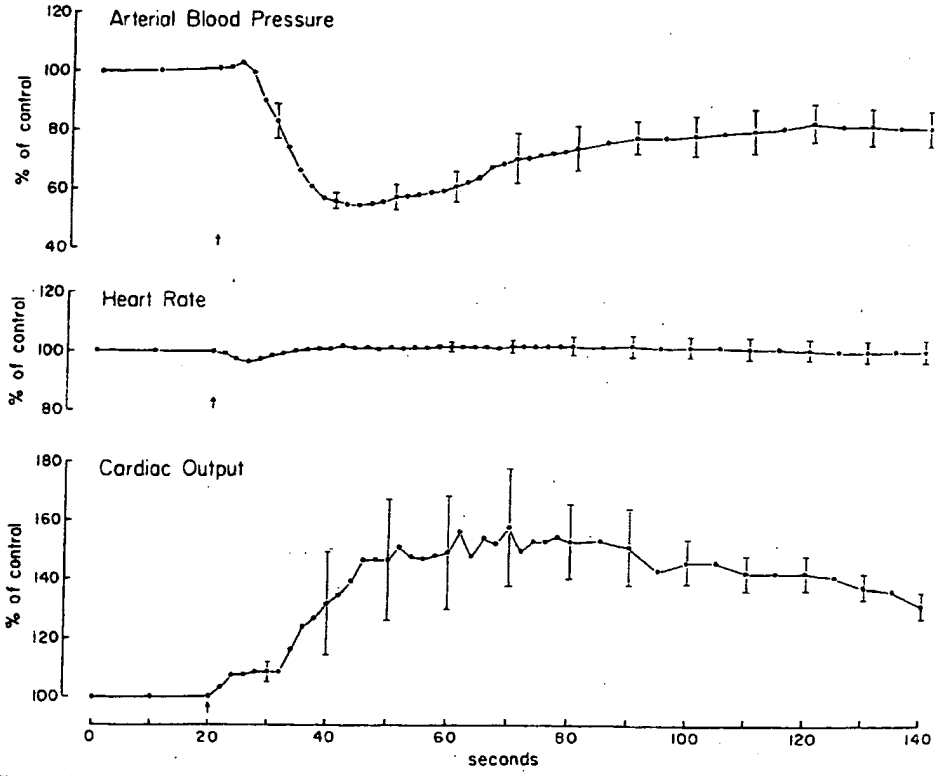



Fig. 4. Time course of the effects of right atrial injection of histamine (10–33 μg/kg) on arterial blood pressure, heart rate and cardiac output. Data are reported as means ± S.E. from 5 cats. Histamine was injected at the arrow.

*Effects of histamine on c*
blood pressure after abo
to its control value aft
beginning about 10 seco
significantly 15 seconds
approximately 25 sec, a
output gradually returne
creased, RAR activity a
usually occurred at this t
the time course of the n

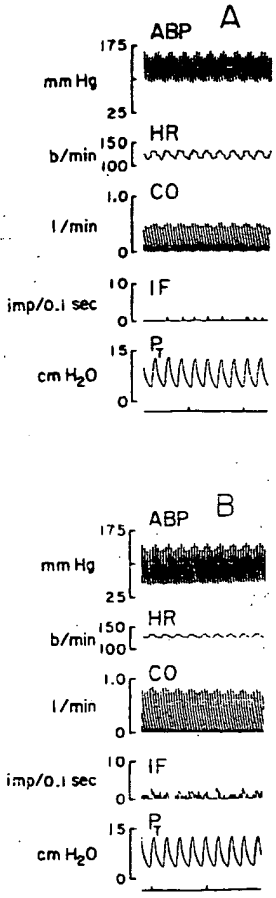
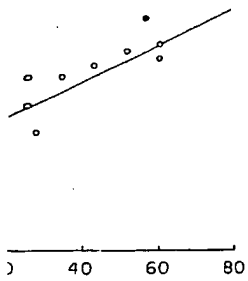


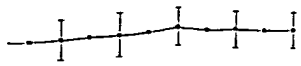

Fig. 5. Relationship betwee
histamine in a cat. A, injectic
A). C, approx 6 min after inj
impulse frequency of a RAR;
output st

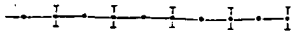
B

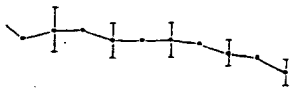


40 60 80

ease in stiffness ($1/C_{DYN}$) produced
he right atrium (●). The data points
cycles with the largest increases in
cles data. Note that RAR activity
by increasing $1/C_{DYN}$.







100 120 140

e (10–33 μg/kg) on arterial blood
S.E. from 5 cats. Histamine was

*Effects of histamine on cardiovascular variables.* Histamine decreased mean arterial blood pressure after about 5 sec following injection; blood pressure returned gradually to its control value after about 30 sec. Pulse pressure also increased significantly beginning about 10 seconds after mean pressure decreased. Cardiac output increased significantly 15 seconds after injection, it reached a peak (50% above control) after approximately 25 sec, and remained elevated for another 30 sec or so. Then cardiac output gradually returned towards the control level (fig. 4). When cardiac output increased, RAR activity also increased (fig. 5), and cardiac modulation of RAR activity usually occurred at this time. The time course of the increase in cardiac output matched the time course of the maximal increase in RAR activity.

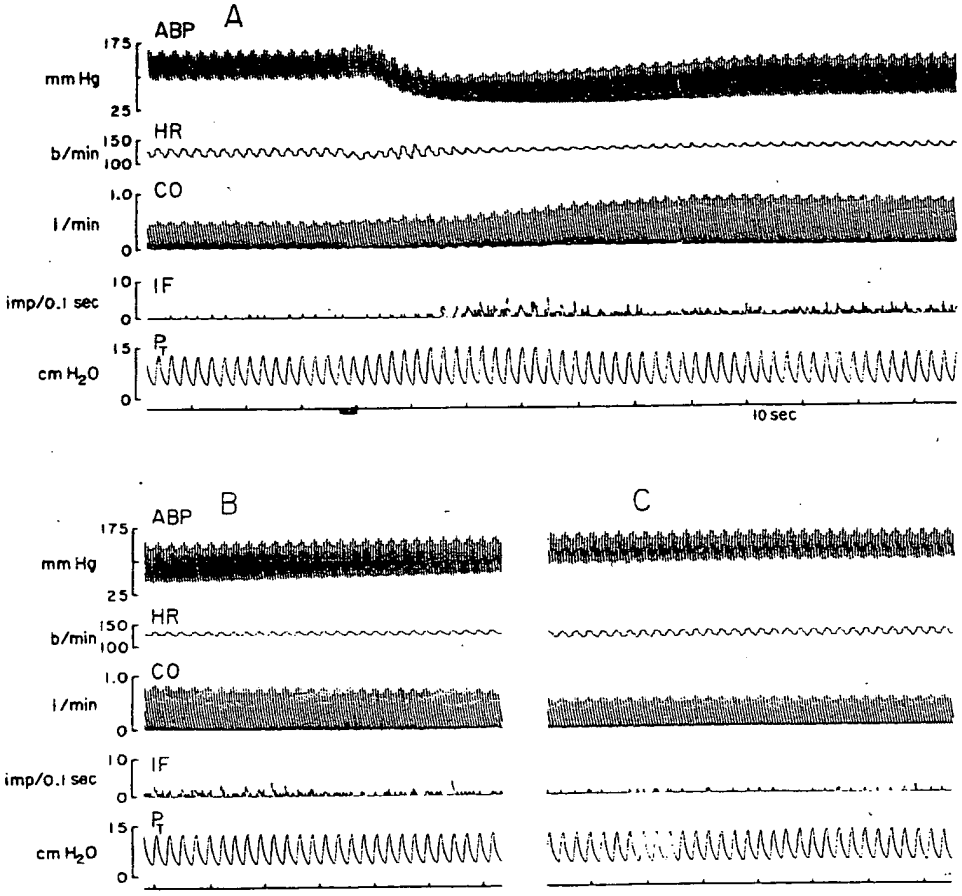



Fig. 5. Relationship between RAR activity and cardiac output in response to right atrial injection of histamine in a cat. A, injection of histamine (33 μg/kg) at the black bar. B, after injection (continuation of A). C, approx 6 min after injection. ABP, arterial blood pressure; HR, heart rate; CO, cardiac output; IF, impulse frequency of a RAR; Pt, tracheal pressure. Note that RAR activity remained elevated while cardiac output stayed high, but tracheal pressure had returned to control.

## Discussion

The afferent properties of RARs have been reviewed (Coleridge and Coleridge, 1986; Sant'Ambrogio, 1987; Pack, 1981). They are mainly mechanosensitive receptors. Changes in lung mechanics, such as increases in tidal volume (Knowlton, 1946), airway flow (Pack and DeLaney, 1983) or decreases in $C_{DYN}$ (Sellick and Widdicombe, 1970; Jonzon *et al.*, 1986; Yu *et al.*, 1987), stimulate RARs. Since histamine alters lung mechanics, it is reasonable to believe that histamine can increase RAR activity by indirect mechanical changes in the lungs.

If RARs were stimulated by histamine entirely as an indirect effect of lung mechanical changes, one would expect to see that their firing pattern in response to histamine resembles the firing pattern in response to a change in lung mechanics with a clear respiratory modulation (Jonzon *et al.*, 1986; Yu *et al.*, 1987; present results). In addition, the time course of RAR activation should correlate with the time course of changes in lung mechanics. When the change in lung mechanics is sustained, the RAR activity will gradually decrease as a result of adaptation (Jonzon *et al.*, 1986; Yu *et al.*, 1987).

On the other hand, if RARs were stimulated by histamine entirely as a direct chemical action, one would expect an irregular discharge with the greatest activity at the onset of activation (without respiratory and cardiac modulation) and a return to control as the chemical is washed away. This pattern resembles the C fiber firing pattern evoked by chemicals (fig. 2). The present results, however, have shown that the response of RARs to right atrial injection of histamine is more complicated.

Our results confirm the results from previous investigators (Mills *et al.*, 1969; Sellick and Widdicombe, 1971; Armstrong and Luck, 1974; Sampson and Vidruk, 1975; Coleridge *et al.*, 1978; Bergren and Sampson, 1982) that histamine increases RAR activity. Our results suggest that the initial increase in RAR activity after histamine injection is due to the change in lung mechanics because: (1) the onset of the increase in RAR activity coincided with the increase in lung stiffness after histamine injection; (2) at this point, the RAR activity was identical to that obtained when lung stiffness was increased by the same amount by changing lung mechanics by removing and then replacing PEEP; (3) at this point the firing pattern of RARs was similar to that obtained when lung stiffness was increased mechanically, *i.e.* both had a clear respiratory modulation.

The maximal response of RARs was relatively late, and did not occur until more than 30 sec after injection. This seems somewhat surprising if the response were simply due to a direct chemical effect and a change of lung mechanics. Although it is possible that regional changes in mechanics could influence receptor activity without being detected as changes in dynamic compliance, there was a clear cardiac modulation of the late response, and the latency of the peak response in RAR activity closely correlated with the latency of the peak response in cardiac output. This suggests that cardiovascular changes produced by histamine played a significant part in the response.

RAR activity has been related to cardiovascular changes, although experiments have

not been designed to iden
activity has cardiac mod
often discharge one or r
1946; Sampson and Vidr
is thought to result fro
(Coleridge and Coleridge
activity and cardiac outp
clusion that histamine c
muscle contraction such

In the present study,
histamine injection can
part of the increase in R
be due to cardiovascula
understanding of the me
we still do not know the
is evidence to suggest th
of sensitization rather t
histamine usually cease
ventilator is switched
background mechanical
in dogs, $PGE_2$, a power
stimulate RARs (Coleri
mechanics are very imp
sensitize the RARs to
histamine alters lung
pattern of RARs is con
effects of increases in

*In conclusion*, the initi
lungs, and that cardiov
RAR activity. RARs ar
increase inspiratory dr
output stimulates RAR
input signals from the c
the body needs.

**Acknowledgements.** We than
supported in part by grants
Association, Kentucky Affil

## References

Armstrong, D.J. and J.C. Lu
*Physiol.* 21: 47–60.

not been designed to identify the nature of the stimulus. It has been mentioned that RAR activity has cardiac modulation. Especially when the lungs are collapsed, the receptors often discharge one or more impulses with each heartbeat (Knowlton and Larrabee, 1946; Sampson and Vidruk, 1975; Sellick and Widdicombe, 1969). The cardiac rhythm is thought to result from the mechanical pulses from the heart and great vessels (Coleridge and Coleridge, 1986). Our present results show a relationship between RAR activity and cardiac output. These results are consistent with other investigators' conclusion that histamine can stimulate RARs by mechanisms other than airway smooth muscle contraction such as by increasing lymph flow (Kappagoda *et al.*, 1988).

In the present study, we did not test to what extent the increase in RAR activity by histamine injection can be attributed to the direct chemical effects. It is reasonable that part of the increase in RAR activity previously ascribed to the direct stimulation might be due to cardiovascular changes. In order to clarify this matter, we need a better understanding of the mechanisms by which histamine activates RARs. Unfortunately, we still do not know the mechanism by which histamine increases RAR activity. There is evidence to suggest that the increase in RAR activity by histamine in dogs is a matter of sensitization rather than outright stimulation, because the RAR activity induced by histamine usually ceases when the ventilator is switched off and resumes when the ventilator is switched on again (Coleridge *et al.*, 1978). This suggests that the background mechanical stimulation is essential to the response. It is noteworthy that in dogs, $PGE_2$, a powerful bronchodilator which is also irritant to the airways, does not stimulate RARs (Coleridge *et al.*, 1978). Once again this suggests that changes in lung mechanics are very important in stimulating RARs. We speculate that histamine may sensitize the RARs to mechanical changes from the cardiopulmonary system. Since histamine alters lung mechanics as well as cardiovascular functions, the response pattern of RARs is complicated. Further experiments need to be done to evaluate the effects of increases in cardiac output on RAR activity.

*In conclusion*, the initial increase of RAR activity is due to mechanical changes in the lungs, and that cardiovascular function may have contributed to the later increase in RAR activity. RARs are believed to elevate the inspiratory off-switch threshold and thus increase inspiratory drive (Coleridge and Coleridge, 1986). If an increase in cardiac output stimulates RARs, it is possible that RARs play a part during exercise to transmit input signals from the cardiopulmonary system to increase the respiratory drive to meet the body needs.

**Acknowledgements.** We thank Jing Juan Zheng for her excellent technical assistance. This investigation was supported in part by grants from the University of Louisville Medical School and the American Heart Association, Kentucky Affiliate.

## References

Armstrong, D.J. and J.C. Luck (1974). A comparative study of irritant and type J receptors in the cat. *Respir. Physiol.* 21: 47–60.

Bergren, D.R. and S.R. Sampson (1982). Characterization of intrapulmonary rapidly adapting receptors of guinea pigs. *Respir. Physiol.* 47: 83–95.

Coleridge, H.M., J.C.G. Coleridge, D.G. Baker, K.H. Ginzel and M.A. Morrison (1978). Comparison of the effects of histamine and prostaglandin on afferent C-fiber endings and irritant receptors in the intrapulmonary airways. *Adv. Exp. Med. Biol.* 99: 291–305.

Coleridge, H.M. and J.C.G. Coleridge (1986). Reflexes evoked from tracheobronchial tree and lungs. In: Handbook of Physiology. Section 3. The Respiratory System. Vol. 2. Control of Breathing. Part 1, edited by N.S. Cherniack and J.G. Widdicombe. Washington, DC, American Physiological Society, pp. 395–429.

Dixon, M., D.M. Jackson and I.M. Richards (1979). The effects of histamine, acetylcholine and 5-hydroxytryptamine on lung mechanics and irritant receptors in the dog. *J. Physiol. (London)* 287: 393–403.

Jonzon, A., T.E. Pisarri, J.C.G. Coleridge and H.M. Coleridge (1986). Rapidly adapting receptor activity in dogs is inversely related to lung compliance. *J. Appl. Physiol.* 61(5): 1980–1987.

Kappagoda, C.T., K. Ravi and K.K. Teo (1988). Effects of histamine (H) infusion on rapidly adapting receptor (RAR) activity and pulmonary lymph flow (PLF). *The Physiologist* 31: A172.

Knowlton, G.C. and M.G. Larrabee (1946). A unitary analysis of pulmonary volume receptors. *Am. J. Physiol.* 147: 100–114.

Mills, J.E., H. Sellick and J.G. Widdicombe (1969). Activity of lung irritant receptors in pulmonary microembolism, anaphylaxis and drug-induced bronchoconstrictions. *J. Physiol.* 203: 337–357.

Pack, A.I. (1981). Sensory inputs to the medulla. *Annu. Rev. Physiol.* 43: 73–90.

Pack, A.I. and R.G. DeLaney (1983). Response of pulmonary rapidly adapting receptors during lung inflation. *J. Appl. Physiol.* 55(3): 955–963.

Sampson, S.R. and E.H. Vidruk (1975). Properties of 'irritant' receptors in canine lung. *Respir. Physiol.* 25: 9–22.

Sampson, S.R. and E.H. Vidruk (1978). Chemical stimulation of rapidly adapting receptors in the airways. *Adv. Exp. Med. Biol.* 99: 281–290.

Sant'Ambrogio, G. (1987). Nervous receptors of the tracheobronchial tree. *Annu. Rev. Physiol.* 49: 611–627.

Sellick, H. and J.G. Widdicombe (1969). The activity of lung irritant receptors during pneumothorax, hyperpnea and pulmonary vascular congestion. *J. Physiol. (London)* 203: 359–381.

Sellick, H. and J.G. Widdicombe (1970). Vagal deflation and inflation reflexes mediated by lung irritant receptors. *Quart. J. Exp. Physiol.* 55: 153–163.

Sellick, H. and J.G. Widdicombe (1971). Stimulation of lung irritant receptors by cigarette smoke, carbon dust, and histamine aerosol. *J. Appl. Physiol.* 31: 15–19.

Vidruk, E.H., H.L. Hahn, J.A. Nadel and S.R. Sampson (1977). Mechanisms by which histamine stimulates rapidly adapting receptors in dog lungs. *J. Appl. Physiol.* 43: 397–402.

Yu, J., J.C.G. Coleridge and H.M. Coleridge (1987). Influence of lung stiffness on rapidly adapting receptors in rabbits and cats. *Respir. Physiol.* 68: 161–176.

*Respiration Physiology*, 79 (19
Elsevier

RESP 01625

# The effects o cholinergic innervate

J.G. Marti

[1]*Meakins-Christie Laboratorie*

**Abstract.** To explore the po studied the effects of exogen preparation to vagal nerve st (ACh). VIP ($2.5 \times 10^{-8}$, 2.5 EFS. Low frequency stimul 20 Hz). VIP inhibited NS ($2.5 \times 10^{-7}$ M) did not sig neutral endopeptidase inhib not affect responses to NS preparation, VIP can modul ganglionic nerves

Acetylch

Vasoactive intestinal p mucosal glands, bron *et al.*, 1981). VIP's pc presence in nerves, an suggestion that it r (Matsuzaki *et al.*, 198 current evidence from system and suggests t system in the airways reactivity and acetylch are co-localized in th

*Correspondence address:* J. Street, Montreal, Quebec,

0034-5687/90/$03.50 © 19

09/919195

| | |
|---|---|
| ACCESSION NUMBER: | 122:154423 CA |
| TITLE: | Ubiquitous receptor: a receptor that modulates gene activation by retinoic acid and thyroid hormone receptors |
| AUTHOR(S): | Song, Ching; Kokontis, John M.; Hiipakka, Richard A.; Liao, Shutsung |
| CORPORATE SOURCE: | The Ben May Inst. Dep. Biochem. Mol. Biol., Univ. Chicago, Chicago, IL, 60637, USA |
| SOURCE: | Proceedings of the National Academy of Sciences of the United States of America (1994), **91**(23), **10809**-13<br>CODEN: PNASA6; ISSN: 0027-8424 |
| PUBLISHER: | National Academy of Sciences |
| DOCUMENT TYPE: | Journal |
| LANGUAGE: | English |

*Proc. Natl. Acad. Sci. USA*
Vol. 91, pp. 10809–10813, November 1994
Biochemistry

# Ubiquitous receptor: A receptor that modulates gene activation by retinoic acid and thyroid hormone receptors

(nuclear receptor/transcriptional regulation/anti-receptor antibody/orphan receptor/prostate cancer)

CHING SONG, JOHN M. KOKONTIS, RICHARD A. HIIPAKKA, AND SHUTSUNG LIAO*

The Ben May Institute and the Department of Biochemistry and Molecular Biology, The University of Chicago, Chicago, IL 60637

*Communicated by Josef Fried, July 11, 1994 (received for review March 24, 1994)*

ABSTRACT **The cDNA for a member of the nuclear receptor family was cloned and named ubiquitous receptor (UR), since UR protein and mRNA are detected in many cell types. Rat UR/human retinoid X receptor α (hRXRα) heterodimers bound preferentially to double-stranded oligonucleotide direct repeats having the consensus half-site sequence AGGTCA and 4-nt spacing (DR-4). Coexpression of UR in COS-1 cells inhibited the stimulation of chloramphenicol acetyltransferase (CAT) reporter gene expression by hRXRα and human retinoic acid receptor α in the presence of all-*trans*-retinoic acid when DR-4 (but not DR-5) was present upstream of the promoter of a CAT reporter gene (DR-4–CAT). UR expression also inhibited the activation of a DR-4–CAT reporter gene by hRXRα and 9-*cis*-retinoic acid or by thyroid hormone receptor β in the presence of thyroid hormone. However, in the absence of 9-*cis*-retinoic acid, UR in combination with hRXRα stimulated DR-4–CAT expression. Coexpression of thyroid hormone receptor markedly reduced this stimulation in the absence of thyroid hormone. UR may play an important role in normal growth and differentiation by modulating gene activation in retinoic acid and thyroid hormone signaling pathways.**

In vertebrates, cellular responses to vitamin $D_3$, retinoids, and steroid and thyroid hormones are dependent on a group of specific nuclear receptors belonging to a superfamily of ligand-activated transcription factors (1). Nuclear receptors modulate the transcription of specific genes by interacting with specific DNA sequences termed hormone response elements (HREs), often located upstream of target genes (2). Whereas steroid hormone receptors interact as homodimers with their cognate HREs arranged as palindromic repeats of 6 nt with 3-nt spacing, members of the thyroid hormone/retinoid receptor subfamily bind most efficiently to an HRE as heterodimers with the retinoid X receptor (RXR) and utilize response elements arranged as direct repeats (DRs) (2, 3). Different receptor/RXR heterodimers have different preferences for the spacing of these DRs (4, 5). The nature of the DNA flanking these half-sites also is important in determining the specificity of a response element (6). Homodimers of the thyroid hormone/retinoid receptor family are also able to modulate transcriptional activity in different ways than their heterodimeric forms (7). The effect of these homo- and heterodimeric receptors on transcriptional activity also depends on their occupancy by specific ligands (8). The complexity of this network of interacting factors is increasing with the discovery of new members of this superfamily of nuclear receptors, many of which are called orphan receptors, since they lack known ligands. An interplay of receptors, ligands, response elements, and yet-to-be-discovered factors may ultimately control the activity of these transcriptional factors and ensure the appropriate cellular responses during development and in the adult.

We report here our discovery of a member of the nuclear receptor family of transcription factors that we have named ubiquitous receptor (UR),† because of its wide tissue distribution. UR is not an isoform of any known receptor and interacts with the response elements and network of receptors in the thyroid receptor (TR)/retinoic acid receptor (RAR) subfamily.

## MATERIALS AND METHODS

**Cloning and Sequencing of cDNA.** A cDNA library was constructed in λZAPII (Stratagene) with poly(A)$^+$ RNA from rat vagina and screened with 5′-$^{32}$P-end-labeled oligodeoxynucleotide probes highly homologous to the DNA-binding domain (DBD) of known rat nuclear receptors. These probes were 5′-TT(A/G)AAGAA(A/T)AC(C/T)TTRCAGCT(T/C/G)CCACA-3′, 5′-CT(A/G)AAGAAICCCTTGCAGCCITCACAGGT-3′, 5′-TT(A/G)AAGAA(A/T)AC(C/T)TTGCAGCT(A/T)CCACAIGT-3′, 5′-C(C/T)CC(A/G)TA(A/G)TG(A/G)CAICC(A/T)GA(A/G)GCITCATC-3′, and 5′-AGTGI(A/T)A(A/G)CCIGTGGC(C/T)(C/T)GGTCICCACA-3′ (where I is deoxyinosine). A clone that hybridized to these probes contained a 1.9-kb cDNA insert that coded for a full-length nuclear receptor. A λZAPII cDNA library constructed with poly(A)$^+$ RNA from PC-3 human prostate cancer cells was screened with the 1.9-kb cDNA insert of the rat UR (rUR) clone to identify human UR (hUR) cDNA clones. Sequences were aligned by use of GENEWORKS software (IntelliGenetics). Similarity searches were performed with the BLAST (9) algorithm and data banks at the National Center for Biotechnology Information (Bethesda, MD).

**Northern Blot Analysis.** Northern blot analysis of poly(A)$^+$ RNA was carried out (10) using a 1.6-kb rUR cDNA fragment (nt 368–1899) labeled with $^{32}$P by random priming (11) as the probe.

**Gel Shift DNA-Binding Assays.** rUR and hRXRα were synthesized *in vitro* by rabbit reticulocyte lysate programmed with RNA transcribed from pSG5 vectors (Stratagene) containing the appropriate cDNAs. Lysate containing rUR and/or hRXRα was incubated with the indicated $^{32}$P-labeled double-stranded oligonucleotide in the absence or presence of unlabeled oligonucleotides containing AGGTCA repeats with variable spacing and orientation. The sequences of the sense strand of these nucleotides (5′-to-3′ with response element

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: r, rat; h, human; $T_3$, 3,3′,5-triiodo-L-thyronine; RA, retinoic acid; *t*-RA, all-*trans*-RA; 9*c*-RA, 9-*cis*-retinoic acid; RAR, RA receptor; RXR, retinoid X receptor; TR, thyroid hormone receptor; UR, ubiquitous receptor; DBD, DNA-binding domain; LBD, ligand-binding domain; DR, direct repeat; HRE, hormone response element; CAT, chloramphenicol acetyltransferase.

*To whom reprint requests should be addressed.

†The sequences reported in this paper have been deposited in the GenBank data base [accession nos. U14533 (rat) and U14534 (human)].

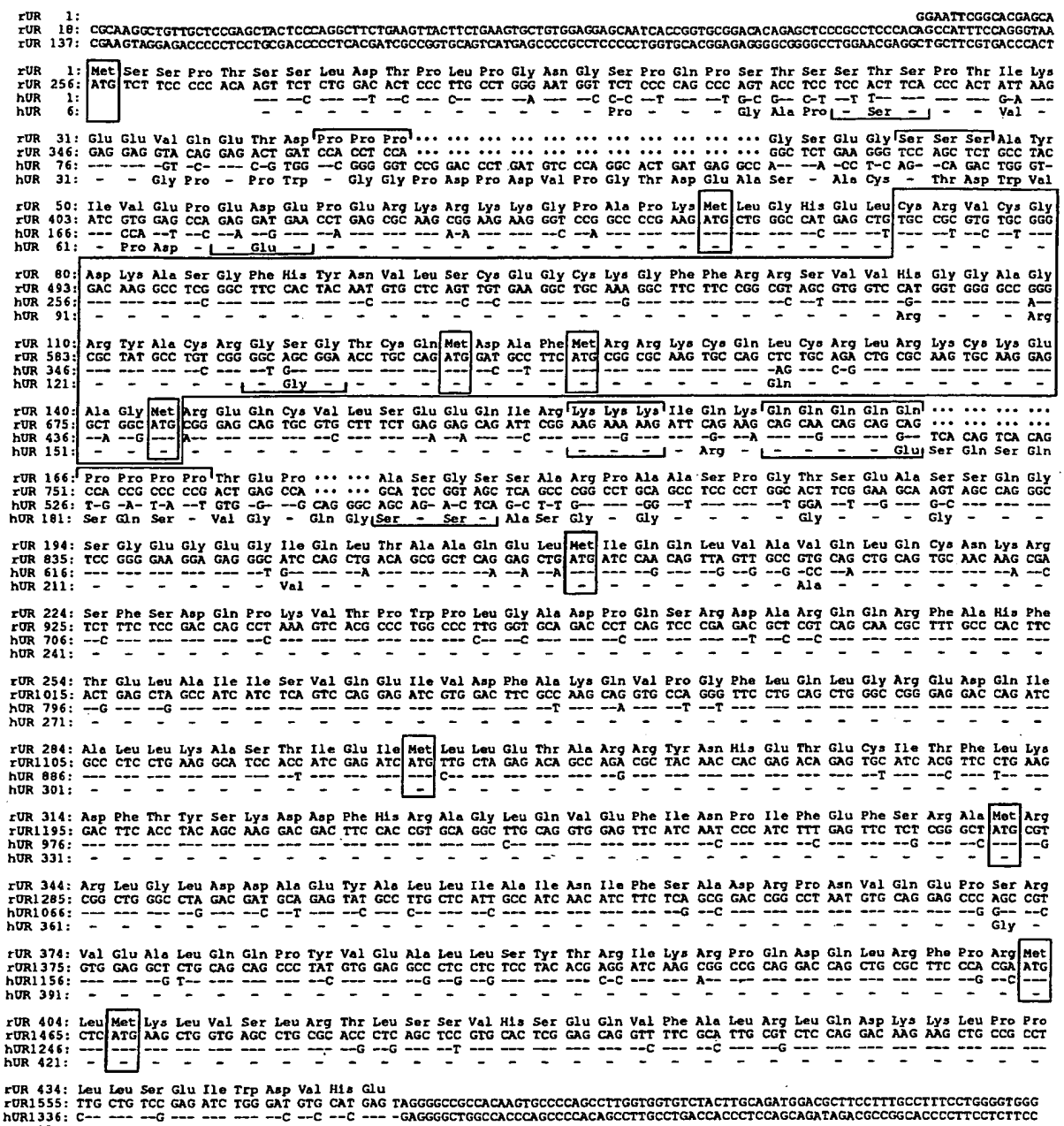


FIG. 1. cDNA sequences and deduced amino acid sequences of rUR and hUR. hUR residues that are identical with rUR residues are shown as hyphens. Gaps introduced to maximize match are indicated by dots. All ATG codons (methionine residues) are boxed. Oligo- or polyamino acid regions are shown by boldface brackets. The putative DBD is boxed.

half-site underlined) were as follows: DR-0, GATCCTCAGGTCAAGGTCAGAAGCT; DR-1, GATCCTCAGGTCAGAGGTCAGAAGCT; DR-2, GATCCTCAGGTCAAGAGGTCAGAAGCT; DR-3, GATCCTCAGGTCAAGGAGGTCAGAAGCT; DR-4, GATCCTCAGGTCACAGGAGGTCAGAAGCT; DR-5, GATCCTCAGGTCACCAGGAGGTCAGAAGCT; DR-6, GATCCTCAGGTCACCAAGGAGGTCAGAAGCT; and pal, GATCAGCTTCAGGTCATGACCTGAGAGCTGATC. For the gel mobility-shift analysis, 2 $\mu$l of programmed lysate was mixed with 20 $\mu$l of binding buffer [10 mM Hepes, pH 7.9/50 mM KCl/2.5 mM $MgCl_2$/2 mM dithiothreitol/10% (vol/vol) glycerol containing poly(dI-dC) (50 $\mu$g/ml) and sonicated salmon testis DNA (250 $\mu$g/ml)] and incubated for 20 min on ice. Labeled probe (20 ng) and

(when indicated) competitors were then added and the mixture was incubated again for 10 min on ice. The DNA–protein complexes were resolved by electrophoresis in 5% polyacrylamide gels containing 23 mM Tris, 23 mM boric acid, and 0.5 mM EDTA, pH 8.0. The gels were run at constant power of 6 W for 3 hr at 4°C, dried, and exposed to XAR-5 Kodak film at −80°C overnight.

**Mammalian Cell Transfection and Chloramphenicol Acetyltransferase (CAT) Assay.** COS-1 cells were plated ($10^6$ cells per 100-mm plate) in Dulbecco's modified Eagle's medium supplemented with either 10% dextran-coated charcoal-stripped or 10% AG1-X8 resin-treated fetal bovine serum (12). The next day, cells were transfected by the calcium phosphate precipitate method (11) with 4 μg of pCH110 (Pharmacia) for β-galactosidase expression, 8 μg of Δ56c-fosCAT (13) reporter plasmid with four tandem copies of DR-3, DR-4, or DR-5 response elements inserted at the *Hin*dIII site, and 4 μg of each kind of nuclear receptor expression vector. For DR-1, the CRBPII-tk CAT vector (14) was used. The pSG5 vector was used for rUR, hRXRα, and hRARα expression, and the pCDM8 vector (Invitrogen) was used for hTRβ expression. Cell transfection, hormone treatments, and CAT assays were performed as described (15). Transfection efficiency was normalized to β-galactosidase activity from cotransfected pCH110 (11). Acetylation of [$^{14}$C]chloramphenicol was quantified after thin-layer chromatography by the AMBIS radioanalytic imaging system (AMBIS Systems). The data shown are the average of duplicates from representative experiments.

**Production of Anti-UR Antibodies.** Antigens were 15-aa peptides from either the amino (UN-15) or the carboxyl (UC-15) terminus of rUR conjugated with keyhole limpet hemocyanin, and an *Escherichia coli* TrpE–rUR (full-length) fusion protein. Polyclonal antibodies were produced in rabbits and affinity purified (16).

## RESULTS AND DISCUSSION

**Structure of rUR.** The nucleotide and deduced amino acid sequences of hUR and rUR cDNAs are shown in Fig. 1. A clone from a rat vagina cDNA library had a 1959-bp insert containing an open reading frame that coded for a protein of 443 aa. The ATG codon at nt 256–258 of the rUR cDNA is in the appropriate context for translation initiation (17), and an in-frame stop codon TGA is present upstream of this codon. The putative protein, with a calculated molecular mass of 49,448 Da, contains a cysteine-rich region, which has a zinc finger motif typical of the DBD of members of the nuclear receptor family.

Sequence comparison with the various receptors of this nuclear receptor family indicates that rUR is most closely related to the *Drosophila* ecdysone receptor, with which it shares 62% amino acid identity in the DBD. The 5 aa (P box) responsible for HRE half-site recognition in other receptors (18, 19) are identical to those of TR and other members of the TR/RAR subfamily. The putative ligand-binding domain (LBD) of rUR is not highly homologous to any other members of the nuclear receptor family.

**Structure of hUR.** All hUR cDNA clones were truncated in the 5′ coding region and lacked DNA coding for 5 aa that are present at the amino terminus of rUR (Fig. 1). Sequence analysis of hUR genomic clones suggests the presence of an intron in this region, based on potential 5′ and 3′ splice sites that conform to consensus splice sites (20). hUR genomic clones were also used as probes to localize the hUR gene to chromosome 19, band q13.3 by fluorescence *in situ* hybridization of normal human metaphase chromosomes (M. M. LeBeau, E. M. Davis, C.S., J.M.K., R.A.H., and S.L., unpublished observation).

The deduced hUR protein sequence (assuming the presence of the missing 5 aa at the amino terminus) has 460 aa with a calculated molecular mass of 50,482 Da and shares nearly 90% homology with rUR (Fig. 1). The percent identity in the DBD as well as the LBD between rUR and hUR is 94%. Most of the amino acid sequence differences lie in the amino-terminal portion of the LBD. There is only one amino acid difference (Ser$^{372}$ of rUR is replaced by Gly at position 389 of hUR) in the 227 aa at the carboxyl-terminal end of the LBD of the two URs.

**UR mRNA Expression and UR Localization in Organs and Cells.** A rUR cDNA probe hybridized to a single prominent 2-kb RNA species on Northern blots of poly(A)$^+$ RNA from heart, liver, kidney, brain, testis, ovary, adrenal, uterus, prostate, vagina, lung, and spleen (Fig. 2). A single band of ≈2 kb was also detected in cultured cells, including the human prostate carcinoma cell lines PC-3 and LNCaP, human skin fibroblasts, BALB/c 3T3 (murine fibroblast line), RPMI 1788 and BJAB (human B-cell lines), and WEHI-231 (a murine immature-B-cell line) (data not shown).

On Western blots UR antibodies reacted with a 50-kDa component in nuclear (but not cytosolic) extracts of rat liver, heart, kidney, prostate, seminal vesicle, small intestine, ovary, and skin. UR was detected predominantly in nuclei by immunocytochemical staining in all organs and cultured cells examined.

**UR Binding to Specific DNA Sequences.** Since the P-box amino acids of the UR DBD were identical to those of TR and other nuclear receptors that bind to AGGTCA half-sites, we analyzed the ability of *in vitro* expressed rUR to bind to oligonucleotides with AGGTCA repeats of different spacing using a gel shift assay. Both rUR monomer and dimer complexes were detected on a series of DRs with 0- to 6-nt spacing. Monomers were present in greatest amount on DR-2 and DR-3, with lesser amounts on DR-1, -4, -5, and -6. Dimer complexes were detected only on DR-3, -4, -5, and -6, with DR-4 having the highest level of bound dimer. Little or no rUR was detected on palindromatic repeats with zero spacing.

Members of the thyroid hormone/retinoid receptor subfamily of nuclear receptor bind to response elements with greater affinity and transactivate genes more effectively when they heterodimerize or are coexpressed with RXR (4,

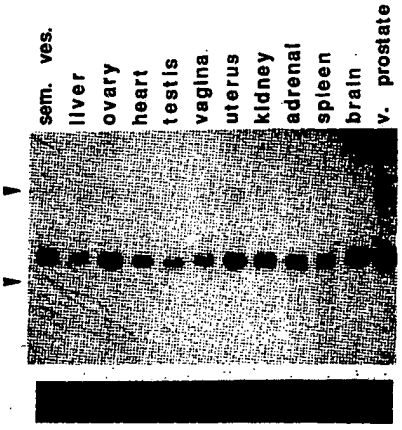



FIG. 2. rUR mRNA expression in various rat tissues. (*Upper*) A Northern blot of poly(A)$^+$ RNA (8 μg) from adult Sprague–Dawley rat tissues was hybridized under stringent conditions with a $^{32}$P-labeled rUR cDNA probe. Arrowheads represent the positions of 28S and 18S ribosomal RNA. sem. ves., Seminal vesicles; v. prostate, ventral prostate. (*Lower*) The membrane was also probed with $^{32}$P-labeled rat glyceraldehyde-3-phosphate dehydrogenase cDNA after the UR probe was stripped off.

21–24). We also found that rUR/hRXR$\alpha$ heterodimers bound to DR sequences with higher affinity than homodimers of rUR or hRXR$\alpha$. Binding of rUR/hRXR$\alpha$ heterodimers to $^{32}$P-labeled DR-4 or DR-5 was inhibited by the addition of nonradioactive DR oligonucleotides (Fig. 3), and DR-4 was the best competitor. rUR/hRXR$\alpha$ heterodimers, therefore, appeared to bind to DR-4 preferentially. Antibodies against the amino terminus of rUR (UN-15) supershifted rUR/hRXR$\alpha$ heterodimers without reducing the total amount of rUR bound to DR-4. Antibodies against the carboxyl terminus of rUR (UC-15) also supershifted some dimers but significantly inhibited the formation of rUR heterodimers bound to DR-4. Therefore, a heptad-repeat leucine zipper structure in the carboxyl-terminal LBD of rUR which is believed to be important in nuclear receptor heterodimerization may be involved in UR heterodimerization and the UC-15 antibody may have blocked this process.

**Reporter Gene Expression in COS-1 Cells Transfected with UR, TR, and RXR.** Expression vectors containing cDNAs for hRXR$\alpha$, hTR$\beta$, and rUR were cotransfected alone or in combination into COS-1 cells along with the DR-4 reporter plasmid, in which four tandemly arranged DR-4 elements were inserted upstream of a heterologous promoter linked to a CAT reporter gene. The CAT activity in cells coexpressing rUR and hRXR$\alpha$ was 4- to 5-fold greater than that in COS-1 cells expressing either rUR or hRXR$\alpha$ alone (Fig. 4*A*). This activity was independent of $T_3$ or 9*c*-RA. Whether rUR/hRXR$\alpha$ activation of the CAT gene required a ligand that was present in culture medium or produced by COS-1 cells is not clear. The rUR/hRXR$\alpha$-dependent CAT activity was markedly reduced by coexpression of hTR$\beta$ in the absence but not the presence of $T_3$. TR in the absence of ligand acts as a transcriptional repressor (8), which may explain the reduction observed. Also, the formation of hTR$\beta$/hRXR$\alpha$ heterodimers may have reduced the formation of rUR/hRXR$\alpha$ heterodimers and utilization of the DR-4 promoter. In the presence of $T_3$, hTR$\beta$ expression alone gave high CAT activity, probably utilizing endogenous RXR (6) as a binding partner (Fig. 4*A*). rUR/hRXR$\alpha$ transactivation of DR-4–CAT, however, required the exogenous coexpression of rUR and hRXR$\alpha$. Addition of 9*c*-RA and $T_3$ to cells coexpressing

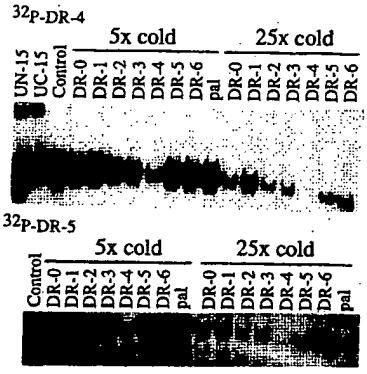



FIG. 3. Relative affinity of rUR/hRXR$\alpha$ heterodimers for various DR oligonucleotides. $^{32}$P-labeled DR-4 (*Upper*) or DR-5 (*Lower*) oligonucleotides were used as probes in a gel shift DNA-binding assay. Nonradioactive ("cold") DR oligonucleotides (5- or 25-fold molar excess over $^{32}$P-DR-4 or $^{32}$P-DR-5) were added as competitors. The control mixture had no competitor. If used, 1 $\mu$g of the indicated antibodies (UN-15 or UC-15) was incubated for 30 min on ice with receptors before the addition of the probe. Only bands representing shifted heterodimer and antibody-supershifted complexes (*Upper*, left two lanes) are shown. The amount of monomer bound to DR oligonucleotides was <20% of the amount of dimer bound to the DR probes. hRXR$\alpha$ alone did not form any protein/DR oligonucleotide complexes under the conditions used.

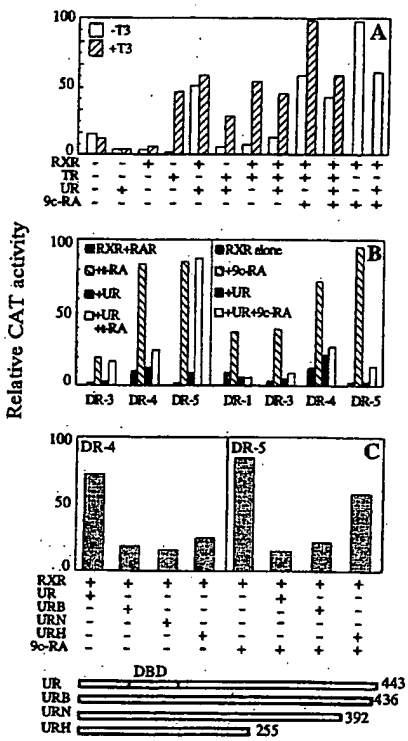



FIG. 4. rUR modulation of hRXR$\alpha$, hRAR$\alpha$, and hTR$\beta$-dependent transactivation of reporter genes. (*A*) Transcriptional activation of a DR-4–CAT reporter plasmid in COS-1 cells by transiently expressed rUR, in combination with hRXR$\alpha$ and hTR$\beta$. 3,3',5-Triiodo-L-thyronine ($T_3$, 100 nM) and/or 9-*cis*-retinoic acid (9*c*-RA, 50 nM) was added to cell cultures as indicated. (*B*) Selective inhibition by rUR of gene transactivation by hRXR$\alpha$/hRAR$\alpha$ heterodimer (*Left*) and hRXR$\alpha$ homodimer (*Right*) in COS-1 cells. All-*trans*-RA (*t*-RA, 1 $\mu$M) t-RA or 9*c*-RA (50 nM) was added to cell cultures as indicated. (*C*) Transcriptional activation and inhibition by carboxyl-terminal truncation mutants of rUR in COS-1 cells cotransfected with a DR-4 or DR-5 reporter gene. Expression vectors containing cDNAs encoding full-length rUR or one of the three carboxyl-terminal deletion mutants shown at the bottom were cotransfected with a hRXR$\alpha$ expression vector.

hRXR$\alpha$ and hTR$\beta$ further induced CAT activity, presumably through the additive activities of hRXR$\alpha$ homodimers and hTR$\beta$/hRXR$\alpha$ heterodimers, although similar high activity was generated with hRXR$\alpha$ and 9*c*-RA alone. This increase in CAT activity was repressed by expression of rUR to the level observed in cells coexpressing only hRXR$\alpha$ and rUR, suggesting that rUR repressed the 9*c*-RA/RXR-dependent CAT activity through the formation of rUR/hRXR$\alpha$ heterodimers, which transactivate the reporter gene less effectively. Also, rUR homodimers bound to DR-4 in gel shift assays (data not shown) but showed little transcriptional activity (Fig. 4*A*) and may have competed with hTR$\beta$/hRXR$\alpha$ heterodimers for the DR-4 response element. Coexpression of rUR repressed $T_3$-dependent hTR$\beta$ stimulation of CAT activity (Fig. 4*A*). This may have been due to the formation of rUR/hTR$\beta$ heterodimers, since we also have observed rUR/hTR$\beta$ (but not rUR/hTR$\alpha$) heterodimers complexed to DR-4 by gel shift analysis.

**UR Modulation of RAR- and RXR-Dependent Transactivation.** Coexpression of hRAR$\alpha$ and hRXR$\alpha$ activated CAT expression from reporter plasmids containing DR-3, DR-4, and DR-5 response elements, although the level of transactivation with DR-3 was only about 20% of that on DR-4 and DR-5 (Fig. 4*B Left*). This *t*-RA-dependent CAT gene activation by hRAR$\alpha$/hRXR$\alpha$ was virtually abolished by coexpres-

sion of rUR in cells transfected with the DR-4 reporter plasmid, but not in cells transfected with the DR-3 or DR-5 reporter plasmids. This specificity may reflect the response element-binding affinity and transcriptional activity of the various homo- and heterodimers present in the transfected cell. rUR homo- and heterodimers bind best to DR-4 but have relatively poor transcriptional activity compared with hRARα/hRXRα, whereas hRARα/hRXRα binds best to DR-5 and has high transcriptional activity. CAT activity induced by 9*c*-RA in cells transfected with a hRXRα expression vector and DR-1, DR-3, DR-4, or DR-5 reporter plasmids was also inhibited by coexpression of rUR (Fig. 4*B Right*). This inhibition might have been due to the formation of rUR/hRXRα heterodimers, which have lower transcriptional activity, and reduction in the level of RXR homodimers which have higher transcriptional activity.

RAR/RXR heterodimers have been shown to transactivate reporter gene expression through DR elements with various nucleotide spacings (25). DR-1 and DR-2 elements have also been reported to be RAR response elements (26). However, the most potent natural RAR response elements are most similar to DR-5 (4). The ability of rUR to inhibit the transcriptional activity of hRARα/hRXRα heterodimers on DR-4 but not DR-5 elements is potentially significant, since this selective inhibition may enable *t*-RA-induced RXR/RAR-dependent transactivation to occur only in genes under the control of a DR-5 element when UR is present.

**Evidence for the Involvement of the Carboxyl Terminus of UR in the Regulation of RXR-Dependent CAT Expression.** Three rUR carboxyl-terminal deletion mutants (URB, URN, and URH; see Fig. 4) were constructed and tested for their ability to modulate gene expression (Fig. 4*C*). The three truncation mutants, including URB, which lacked only 7 aa at the carboxyl terminus, were not as effective as the full-length UR in stimulating CAT gene transactivation in conjunction with RXR. When cells were transfected with the DR-5–CAT reporter plasmid, both UR and URB inhibited 9*c*-RA/hRXRα-dependent induction of CAT activity. This inhibition was much less when URH was used. URH lacks the LBD, which may be important for receptor dimerization.

**Biological Significance.** Although the makeup of the natural response elements for UR, RXR, RAR, and TR in the control regions of various genes is undoubtedly more complex than the synthetic DR sequences used in this study, the interaction of UR with RXR as well as UR modulation of gene transactivation by TR and RAR suggest a mechanism in which a number of nuclear receptors of this subfamily, possibly including some yet to be discovered, interact in a composite fashion to yield a net transcriptional activity in the cell nucleus for a given response element. This net transcriptional activity would additionally be dependent upon the presence of receptor ligands and the particular structure of the response element. The ability of UR to selectively inhibit gene transactivation by RAR/RXR on select response elements is similar to the effect of the orphan receptor COUP-TF, which also acts as a negative regulator of the RA response pathway with certain response elements (27). However, in contrast with UR, COUP-TF does not form heterodimers with RXR in gel shift assays but forms homodimers that compete for binding to response elements. Since UR has the potential to modulate the thyroid hormone signal pathway, it is reasonable to consider whether abnormality in UR function is responsible for some cases of thyroid hormone dysfunction. The ability of UR to restrict transactivation by RAR to specific response elements may indicate a potentially important physiological function for UR.

We thank Dr. E. Fuchs and Dr. L. DeGroot of this university for kindly supplying the hRXRα, hRARα and hTRβ expression vectors. We thank Stephen Andersen and Nara Hojvat-Gallin for excellent technical assistance. This research was supported by Grant CA58073 from the National Cancer Institute and Grants DK41670 and DK37694 from the National Institutes of Health.

1. Evans, R. M. (1988) *Science* **240**, 889–895.
2. Lucas, P. C. & Granner, D. K. (1992) *Annu. Rev. Biochem.* **61**, 1131–1173.
3. Zhang, X.-k. & Pfahl, M. (1993) *Receptor* **3**, 183–191.
4. Umesono, K., Murakami, K. K., Thompson, C. C. & Evans, R. M. (1991) *Cell* **65**, 1255–1266.
5. Naar, A. M., Boutin, J.-M., Lipkin, S. M., Yu, V. C., Holloway, J. M., Glass, C. K. & Rosenfeld, M. G. (1991) *Cell* **65**, 1267–1279.
6. Mader, S., Leroy, P., Chen, J.-Y. & Chambon, P. (1993) *J. Biol. Chem.* **268**, 591–600.
7. Green, S. (1993) *Nature (London)* **361**, 590–591.
8. Lazar, M. A. (1993) *Endocr. Rev.* **14**, 184–193.
9. Altschul, S. F., Gish, W., Miller, W., Myers, E. W. & Lipman, D. J. (1990) *J. Mol. Biol.* **215**, 403–410.
10. Mahmoudi, M. & Lin, V. K. (1989) *BioTechniques* **7**, 331–334.
11. Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab. Press, Plainview, NY), 2nd Ed.
12. Samuels, H. H., Stanley, F. & Casanova, J. (1979) *Endocrinology* **105**, 80–85.
13. Gilman, M. Z., Wilson, R. N. & Weinberg, R. A. (1986) *Mol. Cell. Biol.* **6**, 4305–4316.
14. Mangelsdorf, D. J., Umesono, K., Kliewer, S. A., Borgmeyer, U., Ong, E. S. & Evans, R. M. (1991) *Cell* **66**, 555–561.
15. Kokontis, J., Ito, K., Hiipakka, R. A. & Liao, S. (1991) *Receptor* **1**, 271–279.
16. Hiipakka, R. A., Wang, M., Bloss, T., Ito, K. & Liao, S. (1993) *J. Steroid Biochem. Mol. Biol.* **45**, 539–548.
17. Kozak, M. (1987) *Nucleic Acid Res.* **20**, 8125–8135.
18. Mader, S., Kumar, V., de Verneuil, H. & Chambon, P. (1989) *Nature (London)* **338**, 271–274.
19. Umesono, K. & Evans, R. M. (1989) *Cell* **57**, 1139–1146.
20. Padgett, R. A., Grabowski, P., Konarska, M. M., Seiler, S. & Sharp, P. A. (1986) *Annu. Rev. Biochem.* **55**, 1119–1150.
21. Yu, V. C., Delsert, C., Andersen, B., Holloway, J. M., Devary, O. V., Näär, A. M., Kim, S. Y., Boutin, J.-M., Glass, C. & Rosenfeld, M. G. (1991) *Cell* **67**, 1251–1266.
22. Kliewer, S. A., Umesono, K., Noonan, D. J., Heyman, R. A. & Evans, R. M. (1992) *Nature (London)* **358**, 771–774.
23. Leid, M., Kastner, P., Lyons, R., Nakshatri, H., Saunders, M., Zacharewski, T., Chen, J.-Y., Staub, A., Garnier, J.-M., Mader, S. & Chambon, P. (1992) *Cell* **68**, 377–395.
24. Zhang, X., Hoffman, B., Tran, P. B.-V., Graupner, G. & Pfahl, M. (1992) *Nature* **355**, 441–446.
25. Hall, B. L., Smith-McBride, Z. & Privalsky, M. L. (1993) *Proc. Natl. Acad. Sci. USA* **90**, 6929–6933.
26. Durand, B., Saunders, M., Leroy, P., Leid, M. & Chambon, P. (1992) *Cell* **71**, 73–85.
27. Tran, P., Zhang, X.-k., Salbert, G., Hermann, T., Lehmann, J. M. & Pfahl, M. (1992) *Mol. Cell. Biol.* **12**, 4666–4676.

The EMBO Journal Vol.16 No.7 pp.1656–1669, 1997

# Modulation of retinoic acid sensitivity in lung cancer cells through dynamic balance of orphan receptors nur77 and COUP-TF and their heterodimerization

**Qiao Wu, Yin Li, Ru Liu, Anissa Agadir, Mi-Ock Lee, Yi Liu and Xiao-kun Zhang[1]**

The Burnham Institute, La Jolla Cancer Research Center, 10901 N.Torrey Pines Road, La Jolla, CA 92037, USA

[1]Corresponding author
e-mail: xzhang@ljcrf.edu

**The diverse function of retinoic acid (RA) is mediated by its nuclear receptors, the retinoic acid receptors (RARs) and retinoid X receptors (RXRs). However, the RA response is often lost in cancer cells that express the receptors. Previously, it was demonstrated that the RA response is regulated by the COUP-TF orphan receptors. Here, we present evidence that nur77, another orphan receptor whose expression is highly induced by phorbol esters and growth factors, is involved in modulation of the RA response. Expression of nur77 enhances ligand-independent transactivation of RA response elements (RAREs) and desensitizes their RA responsiveness. Conversely, expression of COUP-TF sensitizes RA responsiveness of RAREs by repressing their basal transactivation activity. Unlike the effect of COUP-TFs, the function of nur77 does not require direct binding of nur77 to the RAREs, but is through interaction between nur77 and COUP-TFs. The interaction occurs in solution and results in inhibition of COUP-TF RARE binding and transcriptional activity. Unlike other nuclear receptors, a large portion of the carboxy-terminal end of nur77 is not required for its interaction with COUP-TF. In human lung cancer cell lines, COUP-TF is highly expressed in RA-sensitive cell lines while nur77 expression is associated with RA resistance. Stable expression of COUP-TF in nur77-positive, RA-resistant lung cancer cells enhances the inducibility of RARβ gene expression and growth inhibition by RA. These observations demonstrate that a dynamic equilibrium between orphan receptors nur77 and COUP-TF, through their heterodimerization that regulates COUP-TF RARE binding, is critical for RA responsiveness of human lung cancer cells.**

*Keywords*: lung cancer/orphan receptors/receptor dimerization/retinoic acid receptors/retinoic acid sensitivity

## Introduction

Retinoic acid (RA) and its natural and synthetic vitamin A derivatives, retinoids, are known to regulate a broad range of biological processes, and are used currently in the treatment of epithelial cancer and promyelocytic leukemia (Gudas *et al.*, 1994; Hong and Itri, 1994). However, retinoid resistance associated with many different types of cancer has prevented retinoids from further application (Warrell *et al.*, 1993; Hong and Itri, 1994). The effects of retinoids are mediated mainly by two classes of nuclear receptors: the retinoic acid receptors (RARs) and the retinoid X receptors (RXRs) (Zhang and Pfahl, 1993; Kastner *et al.*, 1995; Mangelsdorf and Evans, 1995). RARs and RXRs are members of the steroid/thyroid hormone receptor superfamily that also includes a number of orphan receptors whose ligands and function remain to be determined. They modulate the expression of their target genes by interacting as either homodimers or heterodimers with RA response elements (RAREs). Some of the target genes are RARs themselves, including the RARβ gene where a RARE (βRARE) was identified in its promoter region, which mediates up-regulation of RARβ by RA in many different cell types (de The *et al.*, 1990; Hoffmann *et al.*, 1990; Sucov *et al.*, 1990).

Although the expression of RARs and RXRs is essential for the RA response, we and others recently have demonstrated that it is not sufficient to render RA target genes responsive to RA (van der Leede *et al.*, 1993; Zhang *et al.*, 1994; Kim *et al.*, 1995). In lung cancer cell lines, RARs and RXRs are well expressed, but the majority of the cell lines are RA resistant, and RA-responsive genes, such as the RARβ gene, could not be induced by RA (Zhang *et al.*, 1994). The loss of RARβ inducibility by RA is particularly interesting since we have observed recently that up-regulation of the RARβ gene by RA correlates with RA-induced growth inhibition in breast cancer cell lines (Liu *et al.*, 1996) and in lung cancer cell lines (Zhang *et al.*, 1996). In RA-sensitive cancer cell lines, expression of RARβ is strongly induced by RA. In contrast, RA had little effect on RARβ expression in RA-resistant cancer cell lines. *In vivo*, up-regulation of RARβ is associated with clinical response in patients with pre-malignant oral lesions (Lotan *et al.*, 1995). In the course of investigating the mechanism by which RARβ is not induced by RA, we found that the expression of transfected RARs and/or RXRs could not restore RA responsiveness of the βRARE in certain lung cancer cell lines (Zhang *et al.*, 1994). These observations suggest that sensitivity to RA of lung cancer cells is also influenced by factors other than RARs and RXRs. One of the factors known to regulate the RA response is COUP-TF. COUP-TF is encoded by two distinct genes, COUP-TFI (ear-3) (Miyajima *et al.*, 1988; Wang *et al.*, 1989) and COUP-TFII (ARP-1) (Ladias and Karathanasis, 1991), that are orphan members of the nuclear receptor superfamily (Zhang and Pfahl, 1993; Kastner *et al.*, 1995; Mangelsdorf and Evans, 1995). Several studies recently have demonstrated that COUP-TF can repress transcription induced by a number of nuclear receptors including RARs, thyroid hormone receptors (TRs) and vitamin D receptor (VDR)

(Cooney *et al.*, 1992; Kliewer *et al.*, 1992; Tran *et al.*, 1992; Widom *et al.*, 1992), probably due to its competition for DNA binding of the receptors. The binding specificity of COUP-TFs exhibits a strong preference for those bound by retinoid receptors, suggesting that COUP-TFs are probably involved in the regulation of RA target genes.

Nur77 (also known as NGFI-B and TR3) (Chang and Kokontis, 1988; Hazel *et al.*, 1988; Milbrandt, 1988) is another orphan member of the nuclear receptor superfamily. It is induced rapidly by a variety of growth stimuli, including growth factors and phorbol esters (Hazel *et al.*, 1988; Milbrandt, 1988; Williams and Lau, 1993; Lim *et al.*, 1995). How nur77 functions to mediate the growth signaling remains largely unknown. Nur77 binds to its recognition element (NBRE) as a monomer (Wilson *et al.*, 1991). The NBRE consists of the half-site binding motif (AGGTCA) of RAR/TR/VDR with two additional adenine nucleotides at its 5′ end (AAAGGTCA) (Wilson *et al.*, 1991). Interestingly, such sequences are found in the RARβ gene promoter and are located within the βRARE (Perlmann and Jansson, 1995). Investigation of the binding of nur77 on the βRARE demonstrates that nur77 can bind to the βRARE as a heterodimer with RXR (Forman *et al.*, 1995; Perlmann and Jansson, 1995). These observations suggest that nur77 may be involved in the regulation of RARβ gene expression and may function to mediate the interaction between retinoid and growth signalings (reviewed in Leblanc and Stunnenberg, 1995).

In the course of investigating the effect of nur77 on RA-induced RARβ gene expression, we found that nur77 could significantly enhance the transactivation activity of RAREs in a RA and RARE binding-independent manner. By using a variety of approaches, we demonstrate that the effect of nur77 is due to inhibition of COUP-TF RARE binding through direct protein–protein interaction. Transient transfection analysis reveals that COUP-TF RARE binding functions to sensitize the RA responsiveness of RAREs and, conversely, that nur77 desensitizes RAREs through its ability to inhibit COUP-TF RARE binding. In human lung cancer cell lines, loss of RA sensitivity is associated with overexpression of nur77 and/or low expression levels of COUP-TF, and can be restored by introduction and expression of COUP-TF. These results reveals a novel regulatory mechanism established through heterodimerization of orphan receptors nur77 and COUP-TF, that is expected to play an important role in the regulation of retinoid sensitivity of lung cancer cells, and in the cross-talk between growth factors and vitamin A signal transduction pathways in the cells.

## Results

### *Nur77 enhances RARE activity in an RA-independent manner*

We have shown recently that induction of RARβ by RA mediates the growth inhibitory effects of retinoids in human breast cancer and lung cancer cells (Liu *et al.*, 1996; Zhang *et al.*, 1996). RA-induced RARβ expression is mediated mainly by the βRARE in its promoter. To investigate the effect of nur77 on the transactivation of the βRARE, the βRARE-tk-CAT that contains the βRARE cloned into $pBLCAT_2$ (Hoffmann *et al.*, 1990) was used as a reporter gene and was transiently transfected into CV-1 cells. When nur77 expression vector was co-transfected, both all-*trans* RA- and 9-*cis* RA-induced reporter gene activities were enhanced in a concentration-dependent manner (Figure 1). Co-transfection of 200 ng of nur77 expression vector resulted in an ~2-fold increase of the reporter activity when cells were treated with all-*trans* RA. Surprisingly, the basal transcription of the reporter was even greatly increased, with ~5-fold enhancement. The effect of nur77 was specific to the βRARE because addition of nur77 did not show any activity on the parental $pBLCAT_2$ reporter. To investigate the possibility that the nur77 response is due to the presence of an NBRE within the βRARE, we changed two adenines in the spacing region of the βRARE to mutate the NBRE (Figure 1A). The mutations introduced do not affect the consensus half-site binding motifs of RAR/RXR heterodimers. The resulting element (ΔβRARE) was cloned into $pBLCAT_2$ and used as a reporter. When the reporter was analyzed, we observed a similar increase in its basal transcription by co-transfection of nur77 expression vector (Figure 1). This observation suggests that the presence of an NBRE in the βRARE is not essential for the enhancing effect of nur77. To determine whether the enhancing effects of nur77 could be extended to other hormone response elements, reporter constructs containing the tk promoter linked to either TREpal, ApoAI-RARE, CRBPI-RARE, lactoferrin-RARE, CRBPII-RARE, a thyroid hormone-specific response element (MHC-TRE) or a CAT reporter containing the RARβ gene promoter from −60 to +70, including the βRARE (Hoffmann *et al.*, 1990), were transfected into CV-1 cells with or without nur77 expression vector. Similarly to the effect on the βRARE, various degrees of enhancement by nur77 were observed with all the reporter constructs except the reporter containing MHC-TRE (Table I), suggesting that the effect of nur77 may be specific to RAREs. Thus, nur77 can enhance the transactivation of various RAREs in an RA-independent manner.

### *The effect of nur77 on RAREs does not require direct nur77–RARE interaction*

To investigate whether the enhancement of RARE activity by nur77 is due to its binding to the elements, gel retardation assays were performed. When the βRARE was used as a probe, nur77 alone did not exhibit clear binding (Figure 2A). However, a strong complex was formed when nur77 was mixed with RXR. The complex could be upshifted by anti-nur77 antibody and abolished by anti-RXR antibody, demonstrating that the complex represents RXR/nur77 heterodimers. When the ΔβRARE was used as a probe, we did not see any binding of the RXR/nur77 heterodimers (Figure 2B). As a control, RXR/RAR heterodimers formed a strong complex with the element. These data indicate that the integrity of the NBRE within the βRARE is required for efficient RXR/nur77 binding. We also analyzed the binding of nur77 to other RAREs, such as TREpal, CRBPI-RARE, CRBPII-RARE and ApoAI-RARE, and we did not detect any binding of nur77 to these elements either in the presence or absence of RXR or RAR, except a weak RXR/nur77 heterodimer binding to the CRBPII-RARE (data not shown). Together, these results indicate that nur77 enhances the activities of different RAREs via a mechanism that is unlikely to involve a direct nur77/RARE interaction.

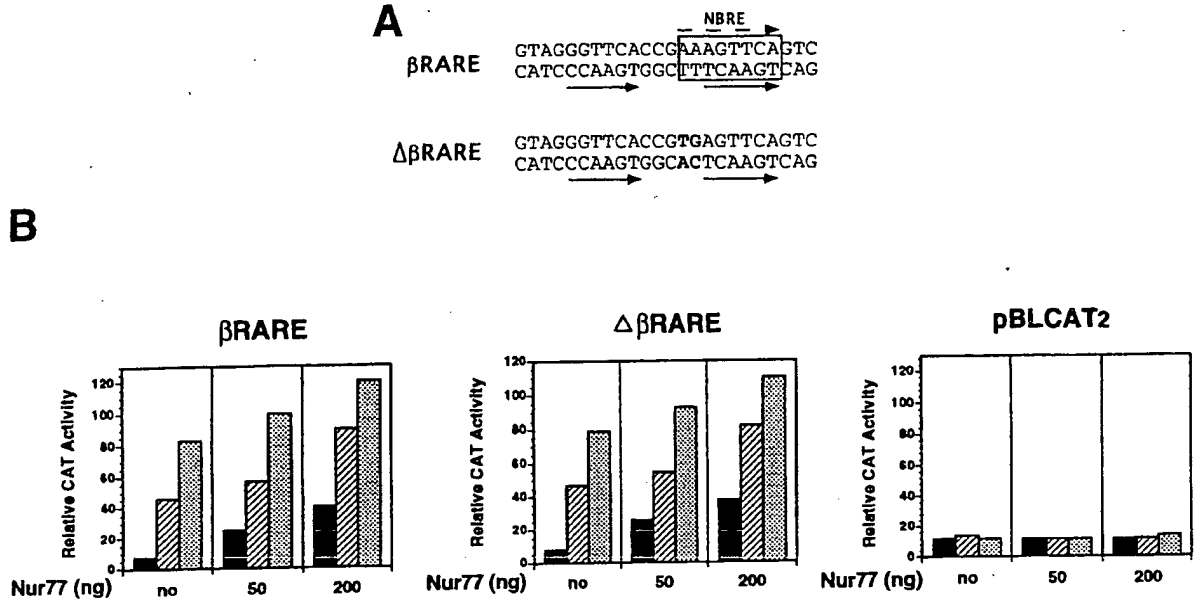



Fig. 1. RA-independent enhancement of RARE activities by nur77. (A) Sequence comparison of βRARE and ΔβRARE. Arrows indicate receptor-binding core motifs. The nur77 binding site (NBRE) is boxed and is also indicated by the dashed arrow. Two nucleotides (in bold) of the NBRE were mutated in the ΔβRARE. (B) Nur77 promotes βRARE and ΔβRARE activities. CV-1 cells were transfected with 100 ng of the indicated CAT reporter gene together with the indicated amounts of nur77 expression vector. Cells were treated with either all-*trans* RA (striped bar), 9-*cis* RA (dotted bar) or no hormone (filled bar), and 24 h later assayed for CAT activity. CAT activity was normalized for transfection efficiency to the corresponding β-gal activity. Data shown represent the means of three independent experiments.

Table I. Nur77 activities on various RAREs and TRE

| Reporter | Fold induction | |
|---|---|---|
| | –Ligand | +Ligand |
| RARβ promoter | 4.5 | 4.1 |
| TREpal | 4.6 | 2.9 |
| ApoAI-RARE | 3.5 | 3.0 |
| CRBPI-RARE | 7.9 | 4.1 |
| CRBPII-RARE | 4.8 | 4.0 |
| Lactoferrin-RARE | 8.3 | 6.6 |
| MHC-TRE | 1.1 | 1.2 |

CV-1 cells were transfected with 100 ng of CAT reporter genes containing the indicated RARE or TRE together with 200 ng of nur77 expression vector. Cells were treated with either all-*trans* RA ($10^{-7}$ M) (for RAREs), thyroid hormone ($10^{-7}$ M) (for MHC-TRE) or no hormone, and 24 h later assayed for CAT activity. CAT activity was normalized for transfection efficiency to the corresponding β-gal activity. Fold induction represents the ratio between relative CAT activity before and after transfection of nur77.

### *Nur77 inhibits COUP-TF DNA binding*

The above data suggest that nur77 may function to repress the activity of an inhibitor on RAREs, thereby alleviating its inhibition. We then investigated the possibility that nur77 affects the binding of COUP-TFs that are known to bind to and restrict transcription of various RAREs (Cooney *et al.*, 1992; Kliewer *et al.*, 1992; Tran *et al.*, 1992; Widom *et al.*, 1992). We first examined the effect of nur77 on COUP-TF binding to the βRARE. COUP-TFI or COUP-TFII formed a strong complex with the βRARE (Figure 3A). However, when nur77 protein was added, the COUP-TF–RARE binding complex was inhibited. The inhibition was very efficient in that a 1 M excess amount of nur77 significantly inhibited the COUP-TF binding and was also specific, as a similar amount of RAR had no effect on the binding (Figure 3A). This result suggests that nur77 may interact with COUP-TF, resulting in formation of nur77/COUP-TF heterodimers that cannot bind to the βRARE. We next investigated whether this interaction could affect nur77/RXR heterodimer binding to the βRARE. When COUP-TFI or COUP-TFII was incubated with nur77 and RXR, the binding of RXR/nur77 to the βRARE was also inhibited efficiently (Figure 3B). A 2 M excess amount of COUP-TFI or COUP-TFII was sufficient to inhibit nur77/RXR heterodimer binding. When a larger amount of COUP-TFI or COUP-TFII was used, the nur77/RXR heterodimer binding was completely inhibited and binding of COUP-TF appeared. Thus, nur77 and COUP-TF can inhibit each other's DNA binding to the βRARE. Nur77 could also inhibit COUP-TF binding to other RAREs, such as TREpal, CRBPI-RARE, CRBPII-RARE, and ApoAI-RARE, although the efficiency of inhibition varied among these elements (data not shown). Together, these data demonstrate that inhibition of COUP-TF DNA binding by nur77 is probably responsible for its enhancement of transactivation activity of RAREs. This is supported by our observation that COUP-TF could not bind to the MHC-TRE (data not shown) which did not show any response to nur77 (Table I).

### *Interaction of nur77 and COUP-TF in solution*

To provide evidence that inhibition of COUP-TF DNA binding by nur77 on RAREs was due to a direct interaction of nur77 and COUP-TF in solution, we first performed an immunoco-precipitation assay using anti-nur77 antibody (Figure 4A). When $^{35}$S-labeled COUP-TFI or COUP-TFII

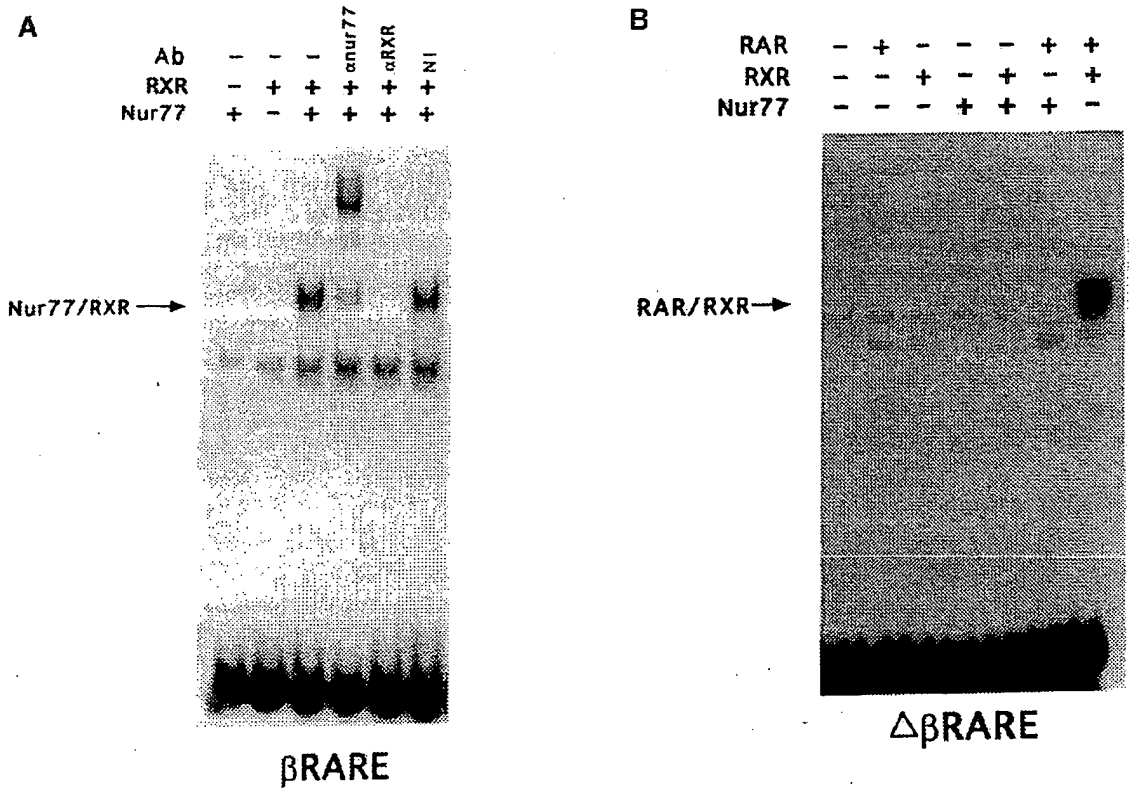



Fig. 2. Binding of nur77 to βRAREs. (A) Nur77 forms heterodimers with RXR on the βRARE. Equal amounts of *in vitro* synthesized nur77 and RXR were incubated alone or together at room temperature for 10 min. The reaction mixtures were then incubated with $^{32}$P-labeled βRARE and analyzed by gel retardation assay. When antibody (Ab) was used, it was incubated with receptor protein for 30 min at room temperature before performing the gel retardation assay. (B) Analysis of nur77 binding to ΔβRARE in the presence or absence of RAR or RXR. An equal amount of nur77 was incubated alone or together with RAR or RXR prior to performing the gel retardation assay using the indicated ΔβRARE as a probe. For comparison, the binding of RAR/RXR heterodimers is shown.

was mixed with nur77, each was precipitated by anti-nur77 antibody. The co-precipitation of COUP-TFI or COUP-TFII by anti-nur77 antibody was specific because neither could be precipitated by non-specific pre-immune serum. In addition, incubation of anti-nur77 antibody with peptide used to generate anti-nur77 antibody prevented its precipitation. To study the interaction further, we cloned nur77 cDNA into pGEX-2T expression vector and expressed a GST–nur77 fusion protein in bacteria. The fusion protein was immobilized on glutathione-Sepharose beads, and mixed with either $^{35}$S-labeled COUP-TFI or COUP-TFII protein. For comparison, labeled RARα and RXRα were used. As shown in Figure 4B, the labeled COUP-TFI or COUP-TFII protein bound specifically to nur77-immobilized Sepharose beads but not to the control beads, demonstrating the specific interaction between nur77 and COUP-TF in solution. Under the conditions used, we did not observe a clear binding of RARα or RXRα to the nur77-immobilized Sepharose beads, suggesting that interaction between nur77 and COUP-TF in solution is much stronger than nur77–RXR interaction. To study whether nur77 and COUP-TF could interact *in vivo*, we cloned nur77 in-frame into the yeast expression vector pGAD424 that contains the Gal4 activation domain, and COUP-TF into the yeast expression vectors pGBT9 that contains the Gal4 DNA-binding domain (DBD). The resulting vectors, pGBT9/nur77 and pGAD424/COUP-TF, were analyzed for their interaction *in vivo* by the yeast two-hybrid system (Bartel *et al.*, 1993). Transformation of either COUP-TF and empty vector pGAD424, or nur77 and empty vector pGBT9 into Y190 yeast cells could not activate the *LacZ* reporter containing the Gal4-binding site. However, when COUP-TF was transformed together with nur77 the reporter was strongly activated (Figure 5). Thus, nur77 and COUP-TF can interact in intact cells. Together, these data demonstrate that nur77 can inhibit COUP-TF DNA binding through a direct protein–protein interaction.

### *Nur77 regions required for interaction with COUP-TF*

Nuclear receptors are characterized by a highly conserved DBD, a well conserved ligand-binding domain (LBD) located at the carboxy-terminal half of the receptor. In addition to ligand binding, the C-terminal region is critical in receptor homo- and heterodimerization (Zhang and Pfahl, 1993; Kastner *et al.*, 1995; Mangelsdorf and Evans, 1995). To determine whether a similar domain of nur77 is employed in the interaction with COUP-TF, two deletion mutants of nur77, Δnur77-1 and Δnur77-2, were constructed (Figure 6A) and tested for their interaction with COUP-TF by the yeast two-hybrid assay (Figure 6B). In Δnur77-1, a segment

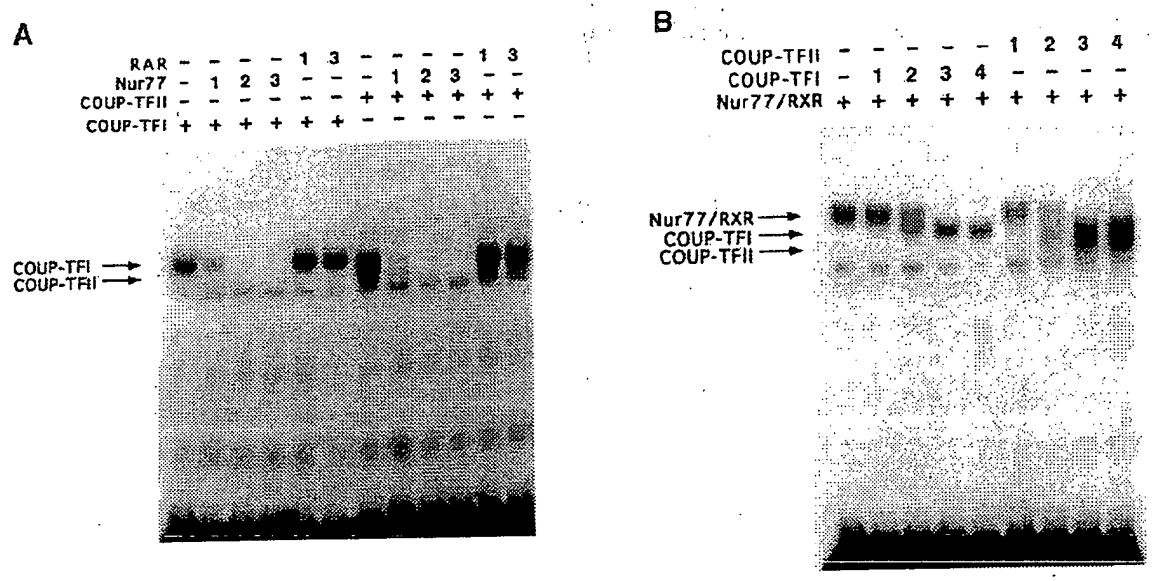



Fig. 3. Mutual inhibition of nur77 and COUP-TF DNA binding. (A) Inhibition of COUP-TF DNA binding on the βRARE by nur77. *In vitro* synthesized COUP-TF was pre-incubated with the indicated molar excess of nur77. Unprogramed reticulocyte lysate was used to maintain an equal protein concentration in each reaction. Following this pre-incubation, the reaction mixtures were incubated with $^{32}$P-labeled βRARE and analyzed by the gel retardation assay. For the control, COUP-TF was also pre-incubated with the indicated molar excess of *in vitro* synthesized RARα protein. (B) Inhibition of nur77/RXR heterodimer binding on the βRARE by COUP-TFs. *In vitro* synthesized nur77 protein was pre-incubated with RXRα in the presence or absence of the indicated molar excess of COUP-TFI or COUP-TFII, and analyzed by gel retardation assay using the βRARE as a probe. Unprogramed reticulocyte lysate was used to maintain an equal protein concentration in each reaction.

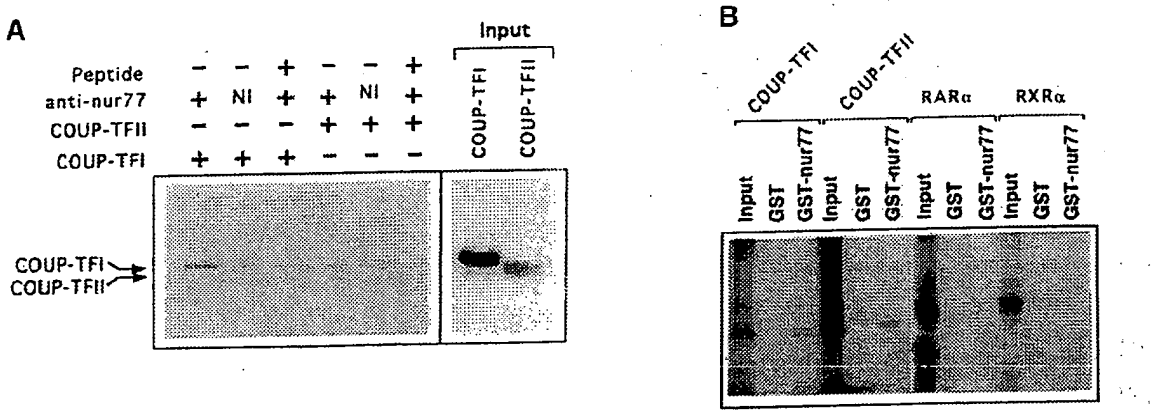



Fig. 4. Direct interaction of nur77 and COUP-TF in solution. (A) Analysis of nur77–COUP-TF interaction by the immunocoprecipitation assay. $^{35}$S-Labeled *in vitro* synthesized COUP-TFI or COUP-TFII was incubated with *in vitro* synthesized nur77. After incubation, either anti-nur77 antibody or non-specific pre-immune serum (NI) was added. In the control, anti-nur77 antibody was pre-incubated with a peptide from which the antibody was generated. The immune complexes were washed, boiled in SDS sample buffer and separated on a 10% SDS–PAGE. The inputs of the labeled COUP-TFI and COUP-TFII are shown for comparison. (B) Analysis of nur77–COUP-TF interaction by the GST pull down assay. To analyze the interaction between nur77 and COUP-TFs further, nur77 protein was synthesized in bacteria using the pGEX-2T expression vector (Pharmacia). The GST/nur77 fusion protein was immobilized on glutathione–Sepharose beads. As a control, the same amount of GST was also immobilized on the beads. $^{35}$S-Labeled COUP-TFI, COUP-TFII, RARα or RXRα was then mixed with the beads. After extensive washing, the bound proteins were analyzed by SDS–PAGE. The input proteins are shown for comparison.

of amino acids from 168 to 468 was removed, whereas in Δnur77-2, 126 amino acids were deleted from the C-terminal end of nur77. Both mutants were cloned in-frame into pGAD424. The resulting vectors, pGAD424-Δnur77-1 and pGAD424-Δnur77-2, were analyzed for their interaction with COUP-TF. Co-expression of Δnur77-1 and COUP-TF did not show any activation of the reporter, suggesting that the deleted region is required for nur77 to interact with COUP-TF. Unexpectedly, the expression of Δnur77-2 together with COUP-TF strongly activated the reporter to a degree similar to that observed with wild-type nur77. Further deletion of 28 amino acids from the

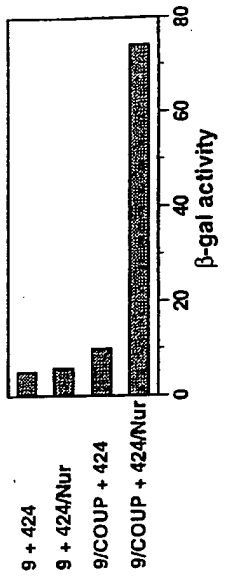



Fig. 5. Nur77 interacts with COUP-TF in yeast. The nur77 and COUP-TFI cDNAs were cloned into the yeast expression vectors pGAD424 (424) and pGBT9 (9), respectively. The resulting expression vectors, 424/nur and 9/COUP, were introduced into Y190 yeast cells as indicated. β-Galactosidase activity was assayed from a yeast strain Y190 containing the LacZ reporter plasmid to study the *in vivo* interaction. The β-galactosidase activity measured with the indicated combinations of yeast expression vectors is shown for comparison.

C-terminal end of Δnur77-2 did not affect its interaction with COUP-TF (data not shown). These data demonstrate that a putative domain is utilized by nur77 to interact with COUP-TF. We also analyzed the domain requirement of COUP-TF. In contrast to nur77, deletion of a region encompassing the DBD (ΔCOUP-TF-1) or 116 amino acids from the C-terminal end (ΔCOUP-TF-2) completely abolished its interaction with nur77, suggesting that both regions are required.

To investigate the interaction of the mutants further, a gel retardation assay was conducted by using the βRARE as a probe (Figure 6C). Similarly to what was observed with wild-type nur77 (Figure 3A), Δnur77-2 effectively inhibited the binding of COUP-TF to the βRARE. In contrast, Δnur77-1 did not show any effect. When the effect of COUP-TF mutants on nur77/RXR heterodimer binding was analyzed, we did not observe any inhibition of nur77/RXR binding. To study whether Δnur77-2 retained its ability to enhance the basal activity of RAREs, Δnur77-2 cloned into the pECE expression vector was transfected into CV-1 cells together with βRARE-tk-CAT (Figure 6D). Like wild-type nur77, Δnur77-2 could also enhance the reporter activity in a RA-independent manner. Similar results were obtained when reporters containing other RAREs were used (data not shown). These gel retardation and transfection data are consistent with the yeast two-hybrid results and suggest that the interaction between nur77 and COUP-TF is mediated by a mechanism that is different from that employed by many other nuclear receptors.

### *Antagonistic effect of nur77 and COUP-TF on modulating the RA sensitivity of RAREs*

The previous demonstration that COUP-TF can inhibit RA-induced activity was based mainly on transient co-transfection assays where COUP-TF might be over-expressed (Cooney *et al.*, 1992; Kliewer *et al.*, 1992; Tran *et al.*, 1992; Widom *et al.*, 1992). We then examined the effect of various concentrations of COUP-TF on the βRARE activity. Co-transfection of larger amounts of COUP-TF expression vector with the βRARE-tk-CAT reporter almost completely inhibited RA-induced reporter activity, consistent with previous results (Cooney *et al.*, 1992; Kliewer *et al.*, 1992; Tran *et al.*, 1992; Widom *et al.*, 1992). However, at low concentrations (1, 5 or 10 ng), COUP-TF either did not affect or even slightly enhanced the RA-induced βRARE activity (Figure 7A). At these concentrations, COUP-TF significantly inhibited the basal activity of the reporter, resulting in an increase of RA-dependent fold induction of the βRARE activity (Figure 7B). Without co-transfection of COUP-TF, a 4-fold induction by RA was seen. However, when 10 ng of COUP-TF expression vector was co-transfected, we observed a 14-fold induction of reporter activity in response to RA. These data are in agreement with observations made previously on the peroxisome proliferator responsive element (PPRE) (Baes *et al.*, 1995) and ApoAI-RARE (Widom *et al.*, 1992), where co-transfection of COUP-TF enhanced the hormonal sensitivity of both responsive elements. Thus, COUP-TF, at appropriate concentrations that are likely to occur in most cells, can enhance the RA sensitivity of the βRARE. To analyze the effect of nur77 on COUP-TF activity, we co-transfected nur77 expression vector together with COUP-TF. As shown in Figure 7A, the inhibition of basal transcription of the βRARE by COUP-TF was alleviated completely when nur77 was co-transfected, resulting in a decrease in RA-dependent fold induction of the βRARE activity (Figure 7B). These data, together with the results shown in Figure 1, demonstrate that nur77 can desensitize the RA responsiveness of RAREs by antagonizing COUP-TF transcriptional activity.

### *Expression of nur77 and COUP-TF in human lung cancer cell lines*

The above observations prompted us to investigate whether expression levels of nur77 and COUP-TF correlate with RA sensitivities observed in various lung cancer cell lines (Figure 7). These cancer cell lines displayed various degrees of RA sensitivity in inducing βRARE despite the fact that RARs and RXRs are well expressed (Zhang *et al.*, 1994; data not shown). Although the degree of RA induction of βRARE activity may also depend on levels of retinoid receptors expressed in the cell lines, we found a perfect correlation between COUP-TF expression and RA induction of βRARE activity (Figure 8). COUP-TF was well expressed in Calu-6, H460, H596, SK-MES-1 and H661 lung cancer cell lines, in which βRARE activity was highly induced by RA. In contrast, COUP-TF transcripts were not detected in other cancer cell lines in which the βRARE was not induced by RA. These observations suggest that the expression of COUP-TF in these cancer cell lines does not repress RA-induced transactivation activity but is required for RA-dependent transactivation

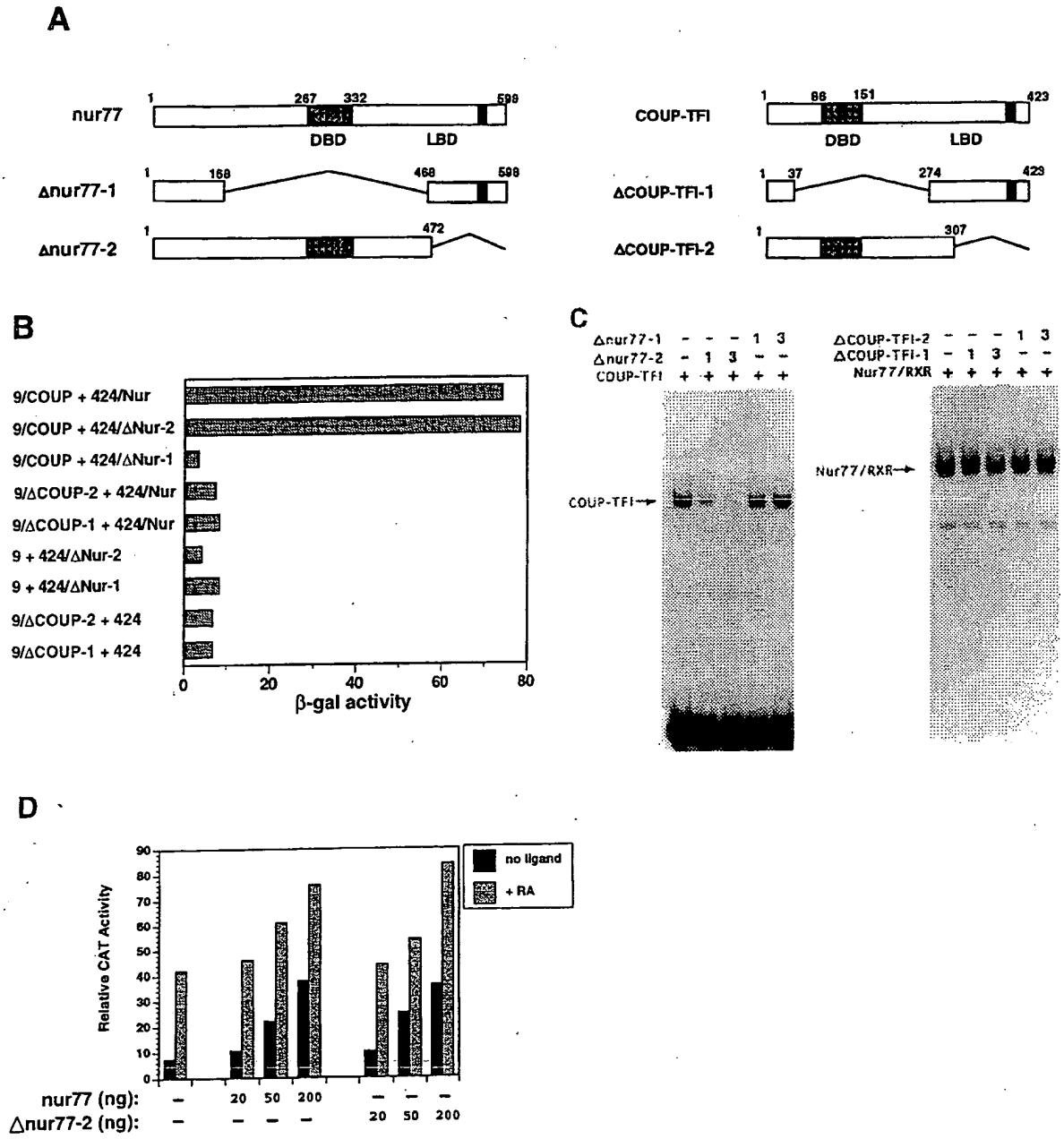



Fig. 6. Domain requirements for nur77–COUP-TF interaction. (A) Schematic representation of the nur77 and COUP-TF deletion mutants. The DNA-binding domain (DBD) and ligand-binding domain (LBD) are indicated. Amino acid numbers are indicated above the bar. (B) Δnur77-2 interacts with COUP-TF in yeast. Δnur77-1 and Δnur77-2 were closed into the pGAD424 vector in-frame and ΔCOUP-TF-1 and ΔCOUP-TF-2 were cloned into pGBT9 in-frame. The resulting expression vectors, 424/Δnur-1, 424/Δnur-2, 9/ΔCOUP-1 and 9/ΔCOUP-2, were introduced into yeast Y190 cells as indicated. b-Gal activity was measured in yeast cells. For comparison, the interaction between wild-type nur77 and COUP-TF is shown. (C) Δnur77-2 inhibits COUP-TF DNA binding. To investigate the interaction between nur77 and COUP-TF further, nur77 deletion mutants were synthesized by *in vivo* transcription–translation, and analyzed for their effect on COUP-TF binding to the βRARE by gel retardation. Similarily, the effect of COUP-TF1 deletion mutants on nur77/RXR binding was analyzed. (D) Δnur77-2 enhances βRARE activity. CV-1 cells were transfected with 100 ng of βRARE-tk-CAT together with the indicated amounts of Δnur77-2 expression vector. Cells were treated with or without $10^{-7}$ all-*trans* RA and assayed for CAT activity. The effect of the wild-type nur77 is shown for comparison.

of the βRARE. Hence, COUP-TF may sensitize βRARE responsiveness to RA through its binding to the element. When the expression of nur77 was analyzed, we found that it was highly expressed in RA-resistant H520 and H292 lung cancer cell lines. Although high levels of nur77 were also observed in RA-sensitive H661 and H460 cell lines, these cell lines expressed significant amounts of COUP-TF, that may counteract the effect of nur77. Under the conditions used, we did not detect expression of nur77 in the RA-resistant H441 cell line. It is likely that factors

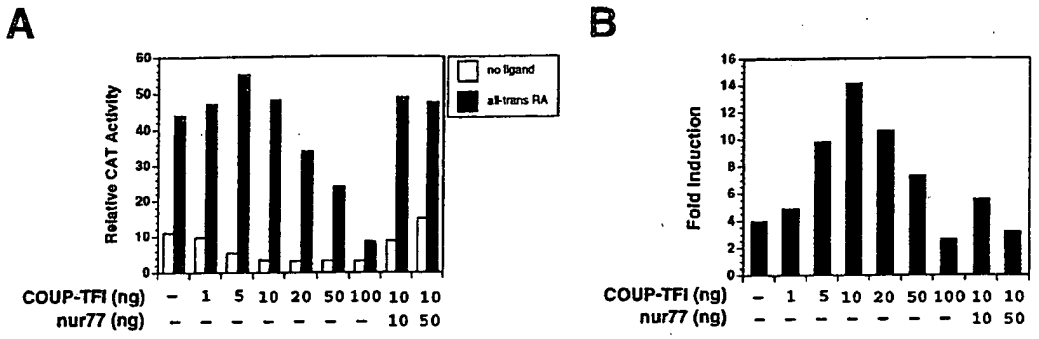



Fig. 7. Modulation of RA sensitivity of βRARE by COUP-TF and nur77. (A) βRARE-tk-CAT was co-transfected with the indicated amounts of nur77 and/or COUP-TF into CV-1 cells. Cells were treated with or without $10^{-7}$ M all-*trans* RA, and 24 h later assayed for CAT activity. Data shown represent the means of three independent experiments. (B) The same data were plotted to indicate the fold activation by RA.

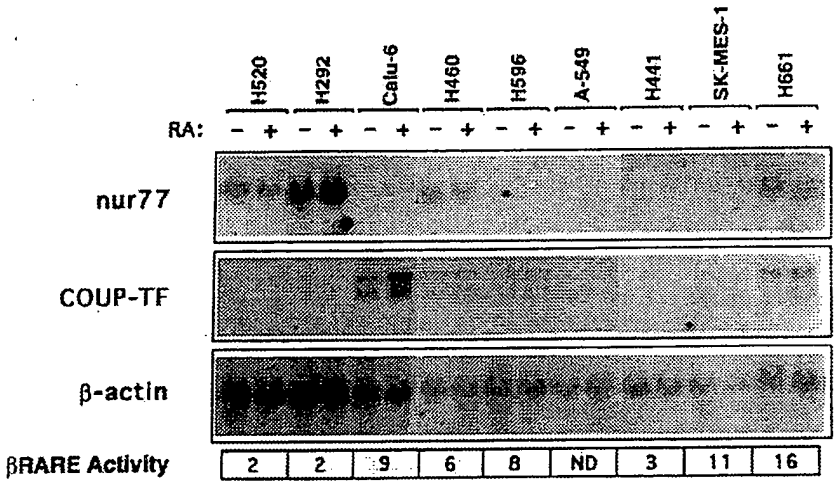



Fig. 8. Expression of COUP-TF and nur77 and RA-dependent βRARE activity in human lung cancer cell lines. Total RNAs were prepared from the indicated human lung cancer cell lines treated with or without $10^{-6}$ M all-*trans* RA for 24 h and analyzed for the expression of COUP-TF and nur77. As a control, the expression of β-actin is shown. βRARE activity represents the fold induction by all-*trans* RA as determined by transient transfection assay using the βRARE-tk-CAT as a reporter.

other than nur77 may be responsible for RA resistance in these cells.

### *Dynamic balance of nur77 and COUP-TF regulates RA sensitivity in human lung cancer cell lines*

In our previous studies, we observed that RARβ was differentially expressed in several human lung cancer cell lines (Zhang *et al.*, 1994). RARβ was not expressed in Calu-6 lung cancer cells but its expression was greatly induced by RA treatment. In contrast, RARβ was highly expressed in H292 lung cancer cells but in an RA-independent manner (Figure 9A). COUP-TF is expressed in RA-sensitive Calu-6 but not in RA-resistant H292 cells, whereas nur77 is expressed in H292 but not in Calu-6 cells. This suggests that relative expression levels of COUP-TF and nur77 may affect expression of the RARβ gene. We therefore analyzed whether co-transfection of nur77 or COUP-TF affects the RA sensitivity of the βRARE in Calu-6 and H292 cells. We first investigated the effect of nur77 in RA-sensitive Calu-6 cells (Figure 9B). When nur77 expression vector was co-transfected together with the βRARE-tk-CAT into the cells, we observed an increase in basal activity and a decrease in RA-dependent fold induction of the reporter. Co-transfection of 50 ng of nur77 expression vector reduced RA-dependent βRARE activity from 7-fold to 2-fold. This result suggests that the high sensitivity of Calu-6 cells to RA may be due to a low expression level of nur77. We next analyzed the effect of COUP-TF on RA-resistant H292 cells (Figure 9C). Co-transfection of COUP-TF expression vector with the βRARE-tk-CAT into the cells reduced basal reporter activity while RA-induced activity was not clearly affected. In the absence of COUP-TF, we did not see a clear effect of RA on βRARE activity. However, when 20 ng of COUP-TF expression vector was co-transfected, we found a 3-fold induction of the βRARE activity by RA. This data demonstrates that loss of RA sensitivity in H292 cells may be due to a low level of COUP-TF in the cells. In addition, nur77 expressed in H292 cells may further inhibit the COUP-TF effect. Thus, a dynamic balance of nur77 and COUP-TF is important in regulating the RA sensitivity of the βRARE in these cancer cells and overexpression of nur77 and/or lack of COUP-TF may be responsible for RA resistance in H292 cells.

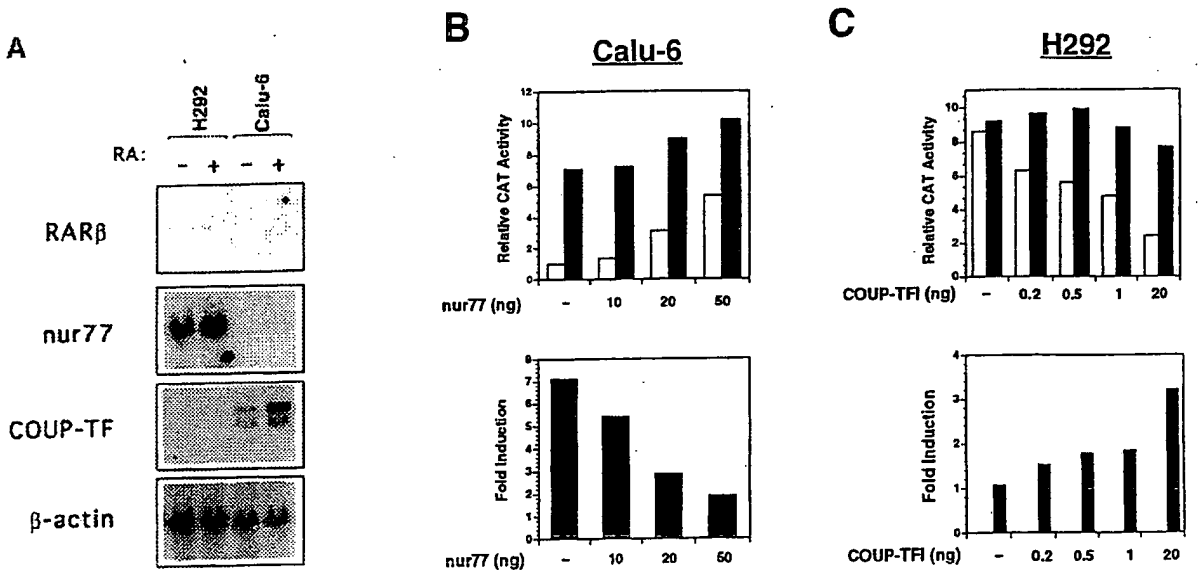



Fig. 9. Modulation of RA sensitivity by COUP-TF and nur77 in human lung cancer cell lines. (A) Effect of RA in inducing RARβ expression in Calu-6 and H292 cell lines. Total RNAs were prepared from Calu-6 or H292 lung cancer cells treated with or without $10^{-6}$ M all-*trans* RA for 24 h and analyzed for the expression of RARβ. For comparison, expression of nur77 and COUP-TF is shown. The expression of b-actin is used as a control. (B) Nur77 decreases RA sensitivity in Calu-6 cells. βRARE-tk-CAT was co-transfected with the indicated amounts of COUP-TFI into Calu-6 cells. The cells were treated with (filled bars) or without (empty bars) $10^{-7}$ M all-*trans* RA for 24 h, and assayed for CAT activity. Data shown represent the means of two experiments. (C) COUP-TF enhances RA sensitivity in H292 cells, βRARE-tk-CAT was co-transfected with the indicated amounts of COUP-TFI into H292 cells. The cells were then treated with (filled bars) or without (empty bars) $10^{-7}$ M all-*trans* RA for 24 h and assayed for CAT activity. Data shown are representative of four independent experiments.

### *COUP-TF or COUP-TF-like protein forms a major complex with βRARE in RA-resistant lung cancer cells*

The above data suggest that COUP-TF may enhance RA sensitivity through its binding to RAREs. To test directly that COUP-TF expressed in RA-sensitive Calu-6 lung cancer cells binds to RARE, nuclear proteins were prepared from Calu-6 cells and RA-resistant H292 cells and analyzed for their RARE binding by gel retardation using the βRARE as a probe. As shown in Figure 10, in addition to several weak complexes, a strong βRARE-binding complex (indicated by the arrow) was observed with nuclear proteins from Calu-6 but not from H292 cells. To determine whether COUP-TF contributed to the βRARE binding, nuclear proteins from Calu-6 cells were incubated with anti-COUP-TF antibody prior to the gel retardation assay. Interestingly, the major βRARE-binding complex was completely upshifted by the anti-COUP-TF antibody, while binding of other weak binding complexes was not affected. Similar results were obtained when CRBPI-RARE was used as a probe (data not shown). Thus, these data clearly demonstrate that the binding of COUP-TF to the βRARE contributes to its effect on the RA sensitivity of the RARE in these lung cancer cell lines.

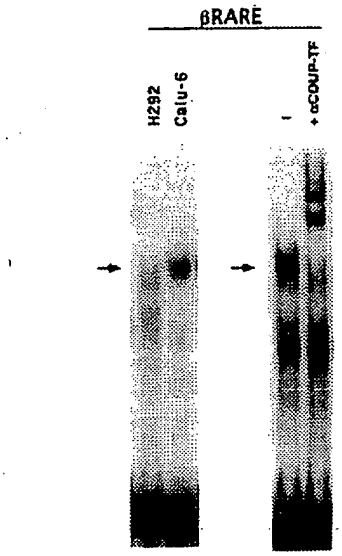



Fig. 10. COUP-TF or COUP-TF-like protein contributes to the βRARE binding activity in an RA-sensitive, COUP-TF-positive lung cancer cell line. Nuclear proteins were prepared from COUP-TF-positive Calu-6 and -negative H292 cells, and analyzed for their DNA binding activity using βRARE as a probe. Nuclear proteins from Calu-6 cells were also analyzed for the effect of anti-COUP-TF antibody. The arrow indicates the binding complex(es) present in Calu-6 but not in H292 cells.

### *Stable expression of COUP-TF restores RA sensitivity in RA-resistant human lung cancer cells*

The observations that nur77 and COUP-TF are differentially expressed in RA-sensitive Calu-6 and RA-resistant H292 cells (Figure 8) and that they can antagonize each other's transcriptional activity (Figure 9) suggest the possibility that constitutive expression of RARβ in H292 cells may be due to overexpression of nur77 and lack of COUP-TF in the cells. To test whether expression of COUP-TF could antagonize the effect of nur77 and

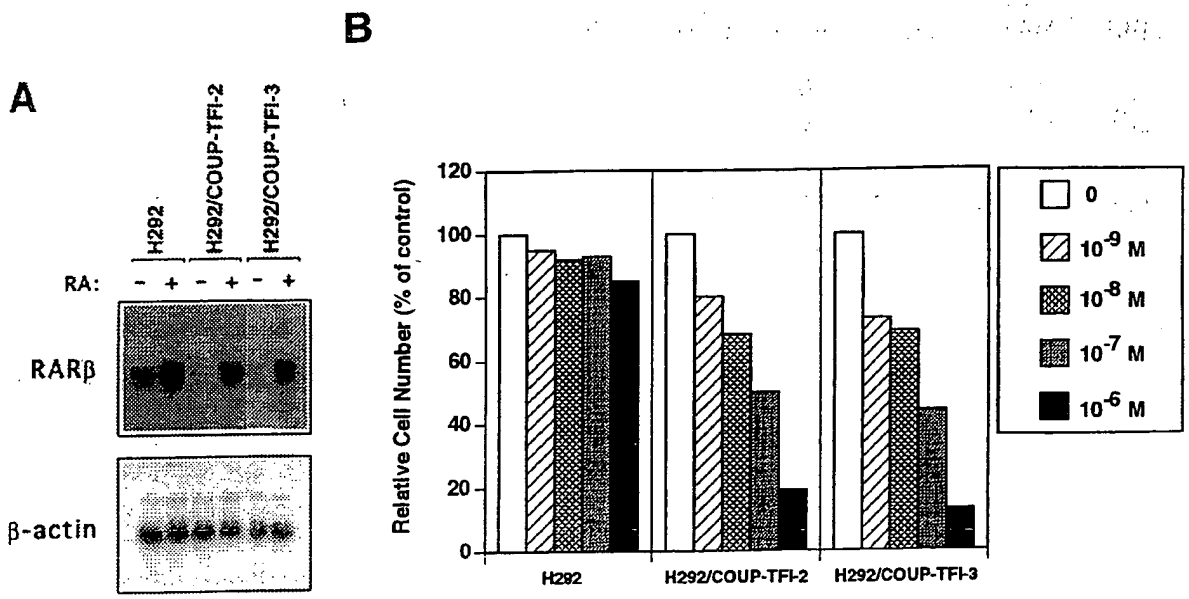



Fig. 11. Stable expression of COUP-TF in RA-resistant H292 cells restores their RA sensitivity. (A) Expression of the RARβ gene in H292 and stable clones. Total RNAs were prepared from Calu-6 and H292 human lung cancer cell lines treated with or without $10^{-6}$ M all-*trans* RA for 24 h and analyzed for the expression of RARβ. In the control, the expression of β-actin is shown. (B) RA-induced growth inhibition in H292 and H292 stable clones that expressed transfected COUP-TF. Cells were seeded at 1000 cells per well in a 96-well plate and treated with the indicated concentrations of all-*trans* RA for 6 days. The numbers of viable cells were determined by the MTT assay.

sensitize RARβ expression responsiveness to RA in H292 cells, we stably expressed COUP-TF in the cells. Two stable clones (H292/COUP-TFI-2 and H292/COUP-TFI-3) that expressed COUP-TF were subjected to analysis of their RARβ gene expression in the absence or presence of RA. In the absence of RA, the level of RARβ expression in these stable clones was largely reduced (Figure 11A), consistent with our transient transfection results (Figure 9C). Surprisingly, when the stable clones were exposed to RA, the reduced level of RARβ was significantly enhanced to the level observed in parental H292 cells (Figure 11A). This, again, is consistent with our transient transfection data (Figure 9C), and provides strong evidence that appropriate levels of COUP-TF expression do not inhibit RA-induced βRARE activity. RA did not significantly inhibit the growth of parental H292 cells (Figure 11B). However, it could now strongly inhibit the growth of the stable clones, with ~85% inhibition observed when the cells were treated with $10^{-6}$ M RA for 6 days (Figure 11B). Thus, the expression of COUP-TF could sensitize RA responsiveness of RARβ expression and growth inhibition in RA-resistant H292 lung cancer cells by reducing the basal activity of the βRARE in the absence of RA.

## Discussion

The diverse functions of RA are mediated mainly by RARs and RXRs. However, expression of RARs and RXRs is often not sufficient to render cells RA responsive. Here we provide evidence that orphan receptors COUP-TF and nur77 play a critical role in the regulation of RA responsiveness of various RA target genes through their modulation of RARE binding. COUP-TFs bind to a variety of RAREs and sensitize their RA responsiveness. Conversely, nur77 reduces RA sensitivity of RAREs through heterodimerization with COUP-TF, which results in inhibition of COUP-TF binding to RAREs. These observations reveal a novel mechanism that modulates RA responses through heterodimerization of orphan receptors COUP-TF and nur77.

### *COUP-TFs function to sensitize the RA responsiveness of RAREs*

Results from several previous studies demonstrate that COUP-TFs function to inhibit RA-induced transactivation of RAREs (Cooney *et al.*, 1992; Kliewer *et al.*, 1992; Tran *et al.*, 1992; Widom *et al.*, 1992). We demonstrate here that COUP-TFs may also function to sensitize the RA responsiveness of RA target genes by reducing their basal activity. In transient transfection assays in CV-1 (Figure 7) and in lung cancer cells (Figure 9), expression of appropriate amounts of COUP-TF repressed the basal transcription of the βRARE-tk-CAT reporter while it had no effect on RA-induced reporter activity. This results in an increase of RA sensitivity of the βRARE (Figures 7 and 9). In a previous study, Baes *et al.* (1995) also observed that COUP-TF inhibited the basal level of the PPRE in the absence of exogenously added ligands. These observations suggest that the sensitizing effect of COUP-TF may represent a general regulatory mechanism of COUP-TF functions. The sensitizing effect of COUP-TF on RAREs is probably due to its binding to the elements since COUP-TF binds strongly to RAREs *in vitro* (Figure 3). In addition, in RA-sensitive Calu-6 lung cancer cells, COUP-TF formed a strong complex with the βRARE, while such a complex was not seen in RA-resistant H292 lung cancer cells that constitutively express RARβ (Figure 10). Hence, the binding of COUP-TF to RAREs

may prevent them from binding and activation by certain RA-independent activators, such as MB67 that binds and activates the βRARE in an RA-independent manner (Baes *et al.*, 1994).

The notion that COUP-TF functions to maintain the RA sensitivity of RAREs by binding to the elements would require that the binding of COUP-TF be replaced by retinoid receptors once retinoids are available. This would suggest that retinoid receptors, upon binding to RA, gain affinity for RAREs. We demonstrated previously that binding of RXR homodimers to RAREs was promoted by its ligand 9-*cis* RA *in vitro* (Zhang *et al.*, 1992b). Although RA does not show a clear effect on RAR/RXR heterodimer DNA binding *in vitro* (Zhang *et al.*, 1992a,b), it was observed, by using *in vivo* footprinting, that RAR–RXR heterodimers do not occupy the βRARE in the absence of RA in P19 cells (Dey *et al.*, 1994) and that RAR ligands can promote retinoid receptor βRARE binding *in vivo* (Chen *et al.*, 1996). Thus, it is likely that, *in vivo*, the ligand induces conformational changes of retinoid receptors so that they have a higher affinity for RARE, that would allow them to replace COUP-TF RARE binding, and subsequently RA responses. Whether liganded retinoid receptors are capable of replacing COUP-TF binding on a RARE may also depend on the binding affinity of COUP-TF for the RARE and expression levels of COUP-TF. For example, COUP-TF has a relatively low affinity for the βRARE (Tran *et al.*, 1992) so that the binding of COUP-TFs may be easily replaced by liganded retinoid receptors. In contrast, the affinity of COUP-TFs for some other RAREs, such as TREpal, is much higher and the binding of COUP-TF to these RAREs may not be replaced easily by retinoid receptors even though they are complexed with ligands. This may explain our observations that certain RAREs, such as TREpal, could not be activated by RA-induced endogenous receptors in RA-sensitive Calu-6 cells while βRARE is highly activated in the same cells (data not shown). Similarly, endogenous receptors in CV-1 cells are sufficient to activate βRARE but not TREpal (Zhang *et al.*, 1992b). Hence, COUP-TF may act to sensitize certain RAREs to their RA responsiveness while at the same time functioning as a silencer of other RAREs depending on RARE binding affinity and expression levels.

### *Nur77 modulates RARE activity through interaction with COUP-TF*

Nur77 is an immediate-early protein whose expression is induced rapidly by a variety of growth stimuli (Hazel *et al.*, 1988; Milbrandt, 1988; Williams and Lau, 1993; Lim *et al.*, 1995). However, the function of nur77 and its mechanism of action remain largely unknown. In the present study, we show that nur77 can enhance the transcriptional activity of a variety of RAREs in an RA-independent manner (Figure 1). Enhancement of RARE activity does not require a direct interaction of nur77 with RARE, since nur77, alone or in the presence of RAR or RXR, does not bind to RAREs except to the βRARE (Figure 2 and data not shown). Binding of nur77/RXR heterodimers to the βRARE may be an alternative mechanism to activate the βRARE since the heterodimers can be induced by certain RXR-selective retinoids (Forman *et al.*, 1995; Perlmann and Jansson, 1995). Our DNA binding experiments indicate that the effect of nur77 is mediated largely by its inhibition of COUP-TF RARE binding. A 2 M excess of nur77 almost completely inhibited COUP-TF binding on the βRARE when nur77 was pre-incubated with COUP-TF (Figure 3). However, if COUP-TF is pre-bound to the RARE, it becomes relative refractory to the inhibitory action of nur77 (data not shown). The inhibition of COUP-TF RARE binding activity by nur77 is likely to be mediated by direct interaction between nur77 and COUP-TF in solution, as demonstrated by our immunoco-precipitation (Figure 4A) and GST pull down experiments (Figure 4B). By using the yeast two-hybrid assay, we show that the interaction can occur *in vivo* (Figure 5). In a transient transfection assay, nur77 can counteract the effect of co-transfected COUP-TF in CV-1 cells (Figure 7). These observations clearly demonstrate that nur77 exerts its effect on RAREs through interaction with COUP-TF, forming complexes that do not bind to the RAREs.

We used deletion mutants to identify domains in COUP-TF and nur77 responsible for interaction. One surprising result is that a large portion of the putative LBD of nur77 is not required for the interaction (Figure 6). This is unexpected because the C-terminal half of nuclear receptors is essential for homo- and heterodimerization of many nuclear receptors, such as RARs, RXR, T3R and VDR (Zhang and Pfahl, 1993; Kastner *et al.*, 1995; Mangelsdorf and Evans, 1995). Our observation would then suggest that the DBD or adjacent sequences is involved in protein–protein interaction or that they are required for other domains in the receptor to achieve the proper conformation for interaction. On the other hand, the A/B region of nur77 is relatively large as compared with other nuclear receptors and may contain sequences responsible for interaction. A detailed analysis will determine the putative domain in nur77 required for interaction with COUP-TF.

### *Regulation of retinoid sensitivity and RARβ expression in lung cancer cells by COUP-TF and nur77*

The observation that expression of COUP-TF is required to maintain RA sensitivity and that nur77 can antagonize the effect of COUP-TF provides a framework for understanding the retinoid sensitivity in cancer cells. This becomes especially apparent since retinoid resistance is observed frequently in various types of cancer cells despite expression of functional retinoid receptors (van der Leede *et al.*, 1993; Zhang *et al.*, 1994; Kim *et al.*, 1995). In human lung cancer cell lines, RARs and RXRs are well expressed, but many of the cell lines show resistance to RA-induced growth inhibition (Zhang *et al.*, 1996) and RARβ expression (Zhang *et al.*, 1994). Our observation that COUP-TF expression is positively correlated with RA sensitivity in lung cancer cell lines (Figure 8) demonstrates that COUP-TF is required for RA sensitivity in the cells. COUP-TF is also highly expressed in RA-sensitive bladder cancer and breast cancer cell lines (data not shown), suggesting that the effect of COUP-TF is not restricted to lung cancer cells. Our studies also reveal that expression of nur77 is associated with retinoid resistance in lung cancer cells (Figure 8). Since nur77 expression is induced rapidly by growth factors and a cAMP-dependent pathway (Hazel *et al.*, 1988; Milbrandt, 1988; Lim *et al.*, 1995), uncontrolled growth signaling in cancer cells may

lead to overexpression of nur77, that in turn may cause retinoid resistance through inhibition of COUP-TF activity. Hence, the studies described here provide an important mechanism by which retinoid sensitivity is regulated in cancer cells.

We have shown recently that up-regulation of RARβ expression by RA correlates with RA-induced growth inhibition in human breast cancer and lung cancer cell lines (Zhang *et al.*, 1996; Liu *et al.*, 1996). In RA-sensitive cancer cell lines, expression of RARβ is strongly induced by RA. In contrast, RA had little effect on RARβ expression in RA-resistant cancer cell lines (Zhang *et al.*, 1996; Liu *et al.*, 1996). *In vivo*, the clinical response of patients with oral dysplasia to RA is associated with inducibility of RARβ (Lotan *et al.*, 1995). The βRARE present in the RARβ promoter mediates the induction of RARβ by RA (de The *et al.*, 1990; Hoffmann *et al.*, 1990; Sucov *et al.*, 1990), and is activated mainly by RAR/RXR heterodimers (Zhang *et al.*, 1992a; Valcarcel *et al.*, 1994). We have observed previously that RARβ cannot be induced by RA in many human lung cancer cell lines even though RAR and RXR are expressed (Zhang *et al.*, 1994). Our present finding that the relative concentrations of COUP-TF and nur77 are involved in the regulation of RARβ inducibility by RA through their modulation of βRARE activity provides an explanation of retinoid refractoriness in inducing RARβ observed in the lung cancer cell lines. COUP-TF is only expressed in lung cancer cell lines in which the βRARE is highly sensitive to RA (Figure 8), suggesting that it is required for maintaining the sensitivity of the βRARE to RA. The effect of COUP-TF is likely to be mediated by its binding to the βRARE, as demonstrated by our finding that COUP-TF expressed in RA-sensitive Calu-6 lung cancer cells formed a strong βRARE-binding complex that was not observed in RA-resistant H292 cells (Figure 10). The observation that RARβ is highly induced by RA in Calu-6 cells (Figure 9A) indicates that the binding of COUP-TF to the βRARE does not interfere with RA-induced retinoid receptor activity. This is also supported by *in vivo* observations (Reuberte *et al.*, 1993; Lutz *et al.*, 1994) that RARβ is expressed in motor neurons at the time when COUP-TF is expressed. Hence, the expression levels we observed in various cancer cell lines do not function to inhibit RA-induced RARβ expression, but repress RARβ expression in the absence of RA. In this study, we also found that nur77 is highly expressed in RA-resistant lung cancer cell lines (Figure 8). Thus, the loss of RA inducibility of RARβ expression in certain lung cancer cell lines, such as H292, is not due to abnormal expression and function of RARs and RXRs that activate the βRARE, but to lack of COUP-TF and/or overexpression of nur77 that modulate basal levels of RARβ expression. Such a loss of RA sensitivity in lung cancer cells can be restored by expression of COUP-TF, as demonstrated by our transient transfection (Figure 9) and stable transfection (Figure 11) of COUP-TF in RA-resistant H292 cells. Interestingly, an increase in RARβ inducibility by stable expression of COUP-TF is also accompanied by an enhancement of growth inhibition by RA (Figure 11). This observation further supports our previous finding that induction of RARβ by RA is involved in RA-induced growth inhibition in breast cancer cell lines (Liu *et al.*, 1996).

In summary, the studies described here reveal a novel mechanism that regulates RA sensitivity in cancer cells through heterodimerization of nur77 and COUP-TF. Our data demonstrate that a dynamic equilibrium of the two orphan receptors plays a crucial role in the control of inducibility of RARβ expression and growth inhibition by RA in human lung cancer cell lines. Such a mechanism may also be involved in the regulation of the RA sensitivity program during development and in adult life. Since the expression of nur77 is induced by growth signaling (Hazel *et al.*, 1988; Milbrandt, 1988; Lim *et al.*, 1995) while the expression of COUP-TF can be enhanced by RA (Jonk *et al.*, 1994), heterodimerization of nur77 and COUP-TF may mediate 'cross-talk' between growth and vitamin A signalings. Overexpression of nur77 and/or lack of COUP-TF as seen in certain human lung cancer cells may be responsible for RA resistance, and may contribute to cell proliferation and neoplastic transformation by releasing the inhibitory effect of RA on cell growth. Our demonstration that expression of COUP-TF in RA-resistant H292 cells could enhance their RA response provides novel approaches for restoring RA sensitivity in certain RA-resistant cancer cells.

## Materials and methods

### *Cell culture*

CV-1 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS). Calu-6 and SK-MES-1 cells were maintained in minimum essential medium (MEM) supplemented with 10% FCS. H292, H520, H460, H596, H441 and H661 cells were grown in RPMI 1640 supplemented with 10% FCS. A-549 cells were maintained in F12 medium supplemented with 10% FCS.

### *Plasmid constructions*

Nur77 expression vectors pECE-nur77, pBluescript-nur77, pGEX-2T-nur77 and pGAD424-nur77 were constructed by cloning the nur77 cDNA (Chang and Kokontis, 1988) fragment into pECE, pBluescript, pGex-2T and yeast vector pGAD424, respectively. The internal *Stu*I fragment was removed from nur77 to generate Δnur77-1. For construction of ΔCOUP-TFI-1, the internal *Nar*I fragment was removed. The deleted COUP-TF was filled in and religated. To obtain the deletion mutants Δnur77-2 and ΔCOUP-TFI-2, the *Pst*I site in nur77 and the *Sal*I site in COUP-TFI were used to delete the C-terminal fragments. The construction of the reporter plasmids βRARE-tk-CAT, TREpal-tk-CAT, CRBPI-RARE-tk-CAT, CRBPII-RARE-tk-CAT, ApoAI-RARE-tk-CAT, lactoferrin-RARE-tk-CAT and MHC-TRE-HC-CAT has been described previously (Tran *et al.*, 1992; Zhang *et al.*, 1992a,b; Lee *et al.*, 1995). The reporter ΔβRARE-tk-CAT was obtained by inserting one copy of mutated βRARE oligonucleotide (TGTAGGGTTCACACTGAGTTCACTCA) (underlining indicates the mutated nucleotides) into the *Bam*HI site of pBLCAT$_2$ (Luckow and Schutz, 1987). The RARβ promoter (*Sma*I–*Eco*RI fragment) reporter has been described (Hoffmann *et al.*, 1990). The construction of COUP-TFI cDNA in the pRc/CMV vector (Invitrogene, San Diego, CA) followed the procedure described previously (Liu *et al.*, 1996).

### *Preparation of receptor proteins*

Receptor proteins were synthesized by an *in vitro* transcription–translation system using rabbit reticulocyte lysate (Promega) as described previously (Zhang *et al.*, 1992a). The relative amount of the translated proteins was determined using [$^{35}$S]methionine-labeled protein on SDS–PAGE, quantitating the amount of incorporated radioactivity, and normalizing it relative to the content of methionine in each protein.

### *Transient and stable transfection assay*

CV-1 cells were plated at $1\times10^5$ cells per well in a 24-well plate 16–24 h before transfection as described previously (Zhang *et al.*, 1992a). For Calu-6 and H292 cells, $5\times10^5$ cells were seeded in six-well culture plates. A modified calcium phosphate precipitation procedure was used for transient transfection and is described elsewhere (Zhang *et al.*,

1992a). Briefly, 100 ng of reporter plasmid, 150 ng of β-galactosidase expression vector (pCH 110, Pharmacia) and various amounts of nur77 expression vector were mixed with carrier DNA (pBluescript) to 1000 ng of total DNA per well. CAT activity was normalized for transfection efficiency to the corresponding β-gal activity. For stable transfection, the pRc/CMV-COUP-TFI recombinant plasmid was stably transfected into H292 cells using the calcium phosphate precipitation method, and screened using G418 (GIBCO BRL, Grand Island, NY) as described (Liu *et al.*, 1996).

### *Gel retardation assay*

The gel retardation assay using *in vitro* synthesized proteins has been described previously (Zhang *et al.*, 1992a). When interaction of nur77 and COUP-TF was studied, they were incubated on ice for 10 min before performing gel retardation in order to prevent the formation of the COUP-TF homodimer. In most cases, co-translation of nur77 and COUP-TF resulted in much more efficient dimerization of the two proteins. When antibodies were used in the gel retardation assay, 1 μl of anti-nur77 (Santa Cruz Biotech., Inc., Santa Cruz, CA) or 1 μl of anti-RXR (Lee *et al.*, 1995) was incubated with receptor protein at room temperature for 30 min prior to performing the gel retardation assay. The oligonucleotides used for the gel retardation assay have been described elsewhere (Tran *et al.*, 1992; Zhang *et al.*, 1992a,b; Lee *et al.*, 1995).

### *Immunoprecipitation assay*

For the immunoprecipitation assay (Zhang *et al.*, 1992a), 5 μl of reticulocyte lysate containing *in vitro* translated $^{35}$S-labeled COUP-TFI or COUP-TFII were incubated with 20 μl of *in vitro* translated nur77 in 100 μl of buffer containing 50 mM KCl and 10% glycerol for 15 min on ice. The reactions were then incubated with 5 ml of anti-nur77 antibody or non-specific pre-immune serum for 2 h on ice. When the peptide from which anti-nur77 antibody (Santa Cruz Biotech., Inc., Santa Cruz, CA) was generated was used, anti-nur77 antibody was incubated with 5 μl of peptide at room temperature for 30 min before adding to the reaction mixtures. Immunocomplexes were precipitated by adding 40 μl of protein A–Sepharose slurry and mixing continuously in the cold room for 1 h. The complexes were then washed five times with RIPA buffer, resuspended in SDS sample buffer containing 15% β-mercaptoethanol, boiled and resolved by SDS–PAGE.

### *GST pull down assay*

To prepare GST–nur77 fusion protein, the nur77 cDNA was cloned in-frame into the expression vector pGEX-2T (Pharmacia). The fusion protein was expressed in bacteria using the procedure provided by the manufacturer, and was analyzed by gel retardation assay and Western blot (data not shown). To analyze the interaction between nur77 and COUP-TF, the fusion protein was immobilized on glutathione–Sepharose beads. For control, the vector protein (GST) prepared under the same conditions was also immobilized. The beads were pre-incubated with bovine serum albumin (1 mg/ml) at room temperature for 5 min. $^{35}$S-Labeled *in vitro* synthesized receptor proteins (2–5 μl, depending on translation efficiency) were then added to the beads. The beads were then rocked continuously for 1 h at 4°C in a final volume of 200 μl in EBC buffer (140 mM NaCl, 0.5% NP-40, 100 mM NaF, 200 μm sodium orthovanadate and 50 mM Tris, pH 8.0). After washing five times with NETN buffer (100 mM NaCl, 1 mM EDTA, 20 mM Tris, pH 8.0, 0.5% NP-40), the bound proteins were analyzed by SDS–PAGE.

### *Two-hybrid assay*

For the yeast two-hybrid assay, the yeast two-hybrid system from Clontech Inc. (Palo Alto, CA) was used. Nur77 cDNA and deletion mutants were cloned into the yeast expression vector pGAD424 to generate an in-frame fusion with the Gal4 activation domain. COUP-TF cDNA and deletion mutants were cloned into pGBT-9 to produce an in-frame fusion with the Gal4 DBD. The yeast reporter strain Y190 containing a LacZ reporter plasmid with the Gal4 binding site was used for transformation. β-Galactosidase activity was determined following the conditions provided by the manufacturer to assess the interaction between nur77 and COUP-TF.

### *MTT assay*

To determine the effect of all-*trans* RA on the viability of the stable transfectants, cells were seeded at 1000 cells per well in a 96-well plate, and treated with various concentrations of all-*trans* RA for 6 days. Media were changed every 48 h. The number of viable cells was determined by MTT assay as described previously (Liu *et al.*, 1996).

### *Northern blot*

For Northern blot analysis, total RNAs were prepared by the guanidine hydrochloride ultracentrifugation method as described (Zhang *et al.*, 1994). Thirty mg of total RNAs from different cell lines treated with or without $10^{-6}$ M all-*trans* RA were analyzed by Northern blot. RARβ, COUP-TFI or nur77 cDNA were used as probes. To determine that equal amounts of RNA were used, the expression of β-actin was studied.

## Acknowledgements

We thank Dr S.E.Harris for the human nur77 expression vector, and S.Goller for preparation of the manuscript. This work was supported in part by grants to X.-k.Z from the National Institute of Health (CA60988 and CA51933) and from the U.S. Army Medical Research Program (DAMD17-4440).

## References

Baes,M., Gulick,T., Choi,H.-S., Martinoli,M.G., Simha,D. and Moore, D.D. (1994) A new orphan member of the nuclear hormone receptor superfamily that interacts with a subset of retinoic acid response elements. *Mol. Cell. Biol.*, **14**, 1544–1552.

Baes,M., Castelein,H., Desmet,L. and Declercq,P.E. (1995) Antagonism of COUP-TF and PPARα/RXRα on the activation of the malic enzyme gene promoter: modulation by 9-*cis* RA. *Biochem. Biophys. Res. Commun.*, **215**, 338–345.

Bartel,P.L., Chien,C.-T., Sternglanz,R. and Fields,S. (1993) Using the two-hybrid system to detect protein–protein interactions. In Hartley,D.A. (ed.), *Cellular Interactions in Development: A Practical Approach*. Oxford University Press, Oxford, pp. 153–179.

Chang,C. and Kokontis,J. (1988) Identification of a new member of the steroid receptor super-family by cloning and sequence analysis. *Biochem. Biophys. Res. Commun.*, **155**, 971–977.

Chen,J.-Y., Clifford,J., Zusi,C., Starrett,J., Tortolani,D., Ostrowski,J., Reczek,P.R., Chambon,P. and Gronemeyer,H. (1996) Two distinct events in retinoid receptor heterodimer action revealed by ligand synergism. *Nature*, **382**, 819–822.

Cooney,A.J., Tsai,S.Y., O'Malley,B.W. and Tsai,M.-J. (1992) Chicken ovalbumin upstream promoter transcription factor (COUP-TF) dimers bind to different GGTCA response elements, allowing COUP-TF to repress hormonal induction of the vitamin $D_3$ thyroid hormone, and retinoic acid receptors. *Mol. Cell. Biol.*, **12**, 4153–4163.

de The,H., Vivanco-Ruiz,M.M., Tiollais,P., Stunnenberg,H. and Dejean,A. (1990) Identification of a retinoic acid responsive element in the retinoic acid receptor β gene. *Nature*, **343**,177–180.

Dey,A., Minucci,S. and Ozato,K. (1994) Ligand-dependent occupancy of the retinoic acid receptor β2 promoter *in vivo*. *Mol. Cell. Biol.*, **14**, 8191–8201.

Forman,B.M., Umesono,K., Chen,J. and Evans,R.M. (1995) Unique response pathways are established by allosteric interactions among nuclear hormone receptors. *Cell*, **81**, 541–550.

Gudas,L.J., Sporn,M.B. and Roberts,A.B. (1994) Cellular biology and biochemistry of the retinoids. In Sporn,M.B., Roberts,A.B. and Goodman,D.S. (eds), *The Retinoids*. 2nd edn. Raven Press, New York, pp. 443–520.

Hazel,T.G., Nathans,D. and Lau,L.F. (1988) A gene inducible by serum growth factors encodes a member of the steroid and thyroid hormone receptor superfamily. *Proc. Natl Acad. Sci. USA*, **85**, 8444–8448.

Hoffmann,B., Lehmann,J.M., Zhang,X.-k., Hermann,T., Graupner,G. and Pfahl,M. (1990) A retinoic acid receptor-specific element controls the retinoic acid receptor-β promoter. *Mol. Endocrinol.*, **4**, 734–1743.

Hong,W.K. and Itri,L.M. (1994) Retinoids and human cancer. In Sporn,M.B., Roberts,A.B. and Goodman,D.S. (eds), *The Retinoids*. 2nd edn. Raven Press, New York, pp. 597–630.

Jonk,L.J.C., de Jonge,M.E., Pals,C.E.G.M., Wissink,S., Vervaart,J.M., Schoorlemmer,J. and Kruijer,W. (1994) Cloning and expression during development of three murine members of the COUP family of nuclear orphan receptors. *Mech. Dev.*, **47**, 81–87.

Kastner,P., Mark,M. and Chambon,P. (1995) Nonsteroid nuclear receptors: what are genetic studies telling us about their role in real life? *Cell*, **83**, 859–869.

Kim,Y.-H. *et al.* (1995) Retinoid refractoriness occurs during lung carcinogenesis despite functional retinoid receptors. *Cancer Res.*, **55**, 5603–5610.

# ARTICLES

## Suppression of Retinoic Acid Receptor β in Non-Small-Cell Lung Cancer In Vivo: Implications for Lung Cancer Development

*Xiao-Chun Xu, Gabriella Sozzi, Jin S. Lee, J. Jack Lee, Ugo Pastorino, Silvana Pilotti, Jonathan M. Kurie, Waun K. Hong, Reuben Lotan**

***Background:*** **Retinoids, analogues of vitamin A, are required for the normal growth and differentiation of human bronchial epithelium. They are also able to reverse premalignant lesions and prevent second primary tumors in some patients with non-small-cell lung cancer (NSCLC). These effects are thought to result from modulation of cell growth, differentiation, or apoptosis (programmed cell death). When certain retinoid receptors in the cell nucleus (i.e., retinoic acid receptors [RARs] and retinoid X receptors [RXRs]), which mediate most retinoid actions, are suppressed, abnormal activity may result that could enhance cancer development.** ***Purpose:*** **This study was designed to determine whether there are abnormalities in the expression of retinoid receptors in surgical specimens from patients with NSCLC.** ***Methods:*** **Transcripts of nuclear retinoid receptors were detected in formalin-fixed, paraffin-embedded specimens by use of digoxigenin-labeled riboprobes specific for RARα, RARβ, RARγ, RXRα, RXRβ, and RXRγ for in situ hybridization to histologic specimens from 79 patients with NSCLC and as control from 17 patients with non-lung cancer. The quality and specificity of the digoxigenin-labeled probes were determined by northern blotting, and the specificity of the binding of antisense riboprobes was verified by use of sense probes as controls.** ***Results:*** **All receptors were expressed in at least 89% of control normal bronchial tissue specimens from 17 patients without a primary lung cancer and in distant normal bronchus specimens from patients with NSCLC. RARα, RXRα, and RXRγ were expressed in more than 95% of the NSCLC specimens. In contrast, RARβ, RARγ, and RXRβ expression was detected in only 42%, 72%, and 76% of NSCLC, respectively.** ***Conclusions:*** **These data suggest that the expression of RARα, RXRα, and RXRγ is not altered in NSCLC; however, expression of RARβ and possibly also of RARγ and RXRβ is suppressed in a large percentage of patients with lung cancer.** ***Implications:*** **The loss of expression of one or more of these nuclear retinoid receptors may be associated with lung carcinogenesis. [J Natl Cancer Inst 1997;89:624-9]**

Lung cancer is still the leading cause of cancer death. The incidence of this cancer continues to increase in both men and women, and mortality from lung cancer has surpassed that from breast cancer in women. It has been estimated that there will be 178 100 new cases and 160 400 deaths from lung cancers in the United States in 1997 (*1*). Despite advances in therapy, the overall 5-year survival rate of patients with lung cancer is still under 13%. Therefore, the identification and use of novel approaches for the prevention and treatment of lung cancer are urgently needed. One such approach is to use retinoids, structural and functional analogues of vitamin A, for chemoprevention (*2*). Retinoids are suitable for this strategy because they regulate differentiation in airway epithelium (*3*) and suppress carcinogenesis in a variety of animal models for lung cancer (*4,5*). Interestingly, vitamin A deficiency has been associated with increased lung cancer incidence (*6*). Furthermore, certain retinoids suppress premalignant oral lesions and prevent the development of second primary cancers among patients with head and neck and lung cancer (*7,8*).

The regulation of cell growth and differentiation of normal, premalignant, and malignant cells by retinoids is thought to result from their effects on gene expression. These effects are mediated by nuclear retinoid receptors, which are ligand-activated transcription factors and members of the steroid hormone receptor superfamily (*9-11*). Two types of receptors have been identified: retinoic acid (RA) receptors (RARs) and retinoid X receptors (RXRs), which differ in the sequence of the amino- and carboxyl-terminal domains and in retinoid-binding specificity. RXR–RAR heterodimers bind to specific DNA sequences—RA response elements (RAREs)—that are usually lo-

**Affiliations of authors:* X.-C. Xu, R. Lotan (Departments of Tumor Biology and Clinical Cancer Prevention), J. S. Lee, J. M. Kurie, W. K. Hong (Department of Thoracic/Head and Neck Medical Oncology), J. J. Lee (Department of Biomathematics), The University of Texas M. D. Anderson Cancer Center, Houston; G. Sozzi (Division of Experimental Oncology A), U. Pastorino (Division of Chest Surgery), S. Pilotti (Division of Anatomic Pathology and Cytology), Istituto Nazionale Tumori, Milan, Italy.

*Correspondence to:* Reuben Lotan, Ph.D., Department of Tumor Biology, The University of Texas M. D. Anderson Cancer Center, 1515 Holcombe Blvd., Houston, TX 77030.

*See* "Notes" following "References."

cated in the 5′ upstream regions of genes that are regulated by retinoids. Each receptor type includes three subtypes designated α, β, and γ, which exhibit specific and distinct spatial and temporal expression patterns during embryonal development and different distributions in adult tissues. These receptors are thought to regulate the expression of distinct genes (*9-11*).

The association between vitamin A deficiency and cancer incidence suggests that retinoids are physiologic suppressors of carcinogenesis. The development of cancer may, therefore, require the initiated and premalignant cells to escape the control of natural retinoids. One way by which this can be accomplished is by aberrant expression of one or more of the retinoid receptors, which could result in an abrogated retinoid signaling. Indeed, several studies (*12-17*) have demonstrated that RARβ expression is suppressed in cultured lung cancer cell lines and have suggested that the expression of this receptor is associated with suppression of cancer. The suppression of tumorigenicity in cultured human lung squamous cell carcinoma transfected with RARβ supports this contention (*18*). However, these results were obtained by use of cultured human lung cancer cell lines; neither their relationship to expression of receptors in normal lung epithelial mucosa cells nor their relevance to the in vivo status of receptors in lung cancer has been established.

This study was designed to determine whether abnormalities exist in the expression of retinoid receptors in surgical specimens from patients with non-small-cell lung cancer (NSCLC).

## Materials and Methods

### Surgical Specimens

Specimens from 79 patients who had surgery from 1984 through 1992 for NSCLC (Table 1) were used in this study. Of the 79 specimens, 37 were obtained from the Department of Pathology, The University of Texas M. D. Anderson Cancer Center, Houston, and 42 were obtained from the Istituto Nazionale Tumori, Milan, Italy. These specimens were selected from pathology archival material to include, in addition to the tumor, adjacent normal and distant normal bronchial epithelial tissue to enable a comparison between receptor expression in tumor and nontumor tissues. Processing of these specimens included a routine fixation in 10% neutral formalin or Bouin's fixative and embedding in paraffin. All of the specimens were cut into 4-μm sections and stained with hematoxylin–eosin for classification.

Table 1. Clinical data of specimens from non-small-cell lung cancers

| Characteristic | Adenocarcinoma* (n = 41) | Squamous cell carcinoma* (n = 38) |
|---|---|---|
| Median age, y (range) | 63 (45-82) | 63 (40-81) |
| Sex | | |
| Female | 12 (29) | 6 (16) |
| Male | 29 (71) | 32 (84) |
| Stage | | |
| I | 21 (51) | 21 (55) |
| II | 8 (20) | 3 (8) |
| III | 7 (17) | 11 (29) |
| IV | 3 (7) | 1 (3) |
| Unknown | 2 (5) | 2 (5) |
| Grade | | |
| Well differentiated | 6 (15) | 6 (16) |
| Moderately differentiated | 21 (51) | 21 (55) |
| Poorly differentiated | 12 (29) | 10 (26) |
| Unknown | 2 (5) | 1 (3) |
| Smoking history | | |
| Current | 22 (54) | 24 (63) |
| Former | 13 (32) | 10 (26) |
| Never | 5 (12) | 2 (5) |
| Unknown | 1 (2) | 2 (5) |

*Unless otherwise specified, values = number of patients (%).

In addition to cancers, these samples also contained adjacent normal lung and bronchial epithelium (n = 40) or distant normal bronchial epithelium (n = 36). As normal lung controls, we used normal lung specimens that were obtained at the Istituto Nazionale Tumori from 17 patients (eight females and nine males) who had undergone lobectomy for lung metastases from primary cancers in other sites of the body (five breast cancers, six sarcomas, two kidney cancers, two melanomas, one laryngeal cancer, and one colon cancer).

### In Situ Hybridization

A previously described method for nonradioactive in situ hybridization (*19*) was used without modifications to analyze nuclear retinoid receptors in formalin-fixed, paraffin-embedded histologic sections. The quality and specificity of the digoxigenin-labeled probes were determined by northern blotting, and the specificity of the binding of antisense riboprobes was verified by use of sense probes as controls (*19,20*). To determine whether the expression of retinoid receptors is different between specimens from normal bronchus and a lung adenocarcinoma, we analyzed consecutive sections by in situ hybridization. To determine whether the expression of retinoid receptors is different between distant normal bronchial epithelium and NSCLC in the same patient, we used available paired specimens obtained after lobectomy from the resection margin of a subset of the 79 patients with NSCLC. To determine whether the expression of the nuclear retinoid receptors is different between the tumor and its surrounding adjacent normal bronchial tissue, we analyzed the expression of the receptors in specimens from available cases that contained both tissue types in the same histologic section.

### Statistical Analysis

Frequency and summary data are given whenever appropriate. The chi-squared test for equality of proportions between normal and tumor tissues was performed for association of each pair. The McNemar test was performed to determine the association between matched pairs, such as the association between distant or adjacent normal tissues and tumors. All *P* values were generated from two-sided statistical tests.

## Results

### Expression of RARs and RXRs in Normal Bronchial Lung Tissue From Patients With Non-Lung Cancer and in NSCLC Specimens

An analysis of consecutive sections of specimens from normal bronchus and a lung adenocarcinoma revealed that antisense probes for all of the receptors (RARα, RARβ, RARγ, RXRα, RXRβ, and RXRγ) hybridized to epithelial cells in all sections (Fig. 1, A, D, G, J, M, and P), whereas the sense probes failed to hybridize to any of the sections (Fig. 1, B, E, H, K, N, and Q), indicating that the binding of the antisense probe is specific. The binding of the antisense probes to consecutive sections of a lung adenocarcinoma (Fig. 1, C, F, I, L, O, and R) was distinct from the binding to the normal bronchial mucosa in that RARβ and RARγ were either absent or barely detectable; RARα's staining intensity was lower than that in normal control specimens, and only minor changes were detected in the staining of RXRs in normal and carcinoma sections.

The results of an analysis of bronchial specimens from 17 patients without primary lung carcinoma (normal lung) and tumor specimens from 79 patients with NSCLC are shown in Table 2. The most dramatic finding was the loss of RARβ expression in 58% of the specimens (53% of squamous cell carcinomas [SCCs] and 63% of adenocarcinomas). The difference in RARβ expression between normal tissue and NSCLC was statistically significant. Furthermore, about 25% of the NSCLCs also lost RARγ and RXRβ expression. In contrast, the distribution of RARα, RXRα, and RXRγ expression was very similar in normal control and NSCLC specimens. There was no apparent

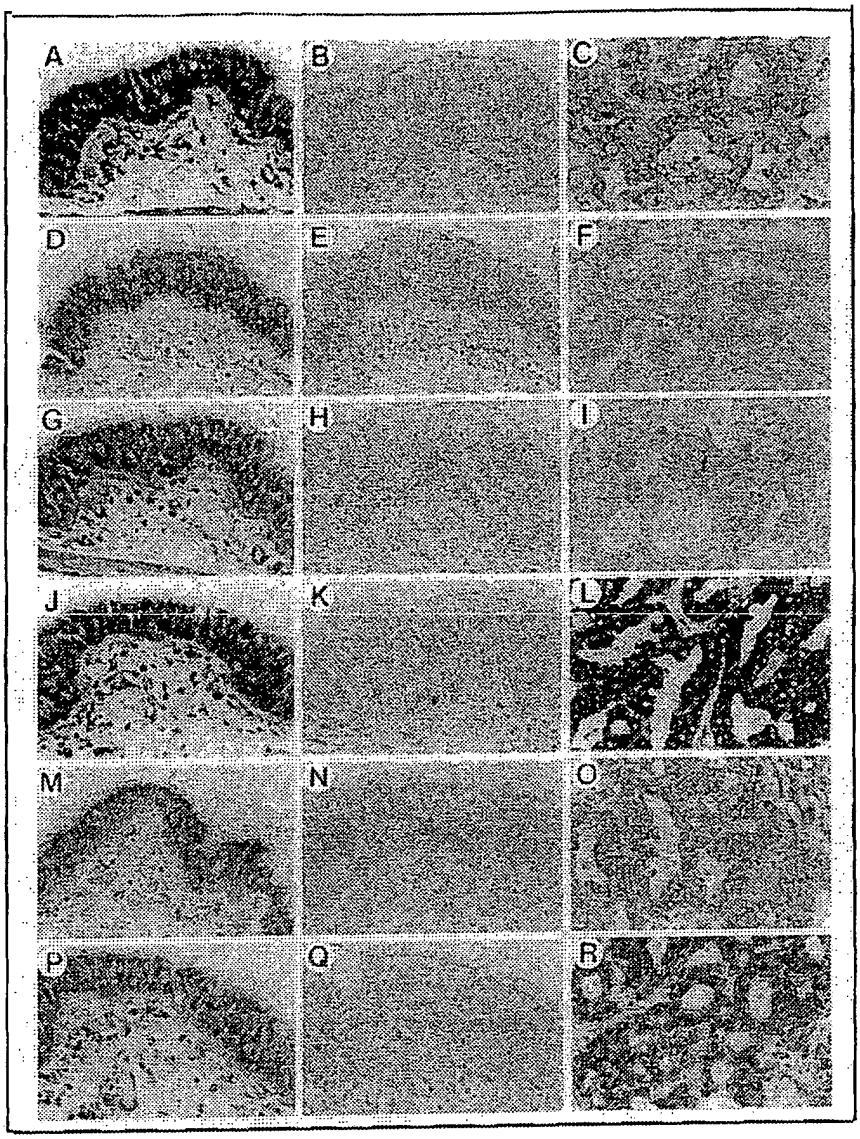



**Fig. 1.** Localization of nuclear retinoic acid receptor (RAR) and retinoid X receptor (RXR) messenger RNAs in sections of surgical specimens from normal bronchus and lung adenocarcinoma by in situ hybridization. Consecutive sections of formalin-fixed, paraffin-embedded normal human lung tissue were hybridized with RARα antisense (A) or sense (B); RARβ antisense (D) or sense (E); RARγ antisense (G) or sense (H); RXRα antisense (J) or sense (K); RXRβ antisense (M) or sense (N); and RXRγ antisense (P) or sense (Q). Consecutive sections of lung adenocarcinoma were hybridized with antisense riboprobes for RARα (C); RARβ (F); RARγ (I); RXRα (L); RXRβ (O); or RXRγ (R).

relationship between the differentiation status of NSCLC and RARβ expression because six of 12 well-differentiated tumors expressed RARβ. Likewise, the loss of RARβ expression was not associated with the stage of disease when stage I was compared with stages II-IV, most likely because this event may have occurred already in premalignant lesions and was maintained throughout the carcinogenesis process, as we had observed in head and neck carcinogenesis (*20,27*). Because only seven of the 79 patients with NSCLC were never smokers and three of them did lose RARβ expression, it was not possible to relate smoking status to RARβ expression. This lack of association was maintained, even after combining never smokers with remote former smokers (quit smoking >2 years ago).

### Expression of RARs and RXRs in Paired Specimens of Distant Normal Bronchial Lung Tissue and NSCLC From the Same Patient

Table 3 shows that more than 90% of the distant normal specimens expressed all six retinoid receptor messenger RNAs

**Table 2.** Expression of RAR and RXR messenger RNAs in normal bronchial epithelia and NSCLC*

| | % positive cases (No. positive/total) | | | |
|---|---|---|---|---|
| Receptor | Normal | NSCLC | SCC† | ADC† |
| RARα | 100 (17/17) | 94.7 (72/76) | 94.4 (34/36) | 95.0 (38/40) |
| RARβ | 88.9 (15/17) | 61.8 (33/79)‡ | 42.4 (18/38)§ | 36.6 (15/41)‖ |
| RARγ | 94.1 (16/17) | 72.4 (55/76) | 74.3 (26/35) | 70.7 (29/41) |
| RXRα | 100 (17/17) | 100 (63/63) | 100 (30/30) | 100 (33/33) |
| RXRβ | 94.1 (16/17) | 76.1 (51/67) | 73.3 (22/30) | 78.4 (29/37) |
| RXRγ | 88.9 (15/17) | 95.2 (40/42) | 100 (18/18) | 91.7 (22/24) |

*RAR = retinoic acid receptor; RXR = retinoid X receptor; NSCLC = non-small-cell lung carcinoma; SCC = squamous cell carcinoma; ADC = adenocarcinoma.

†NSCLC cases can be further categorized into SCCs and ADC.

‡$P = .0005$ between normal and NSCLC.

§$P = .004$ between normal and SCC.

‖$P = .0007$ between normal and ADC.

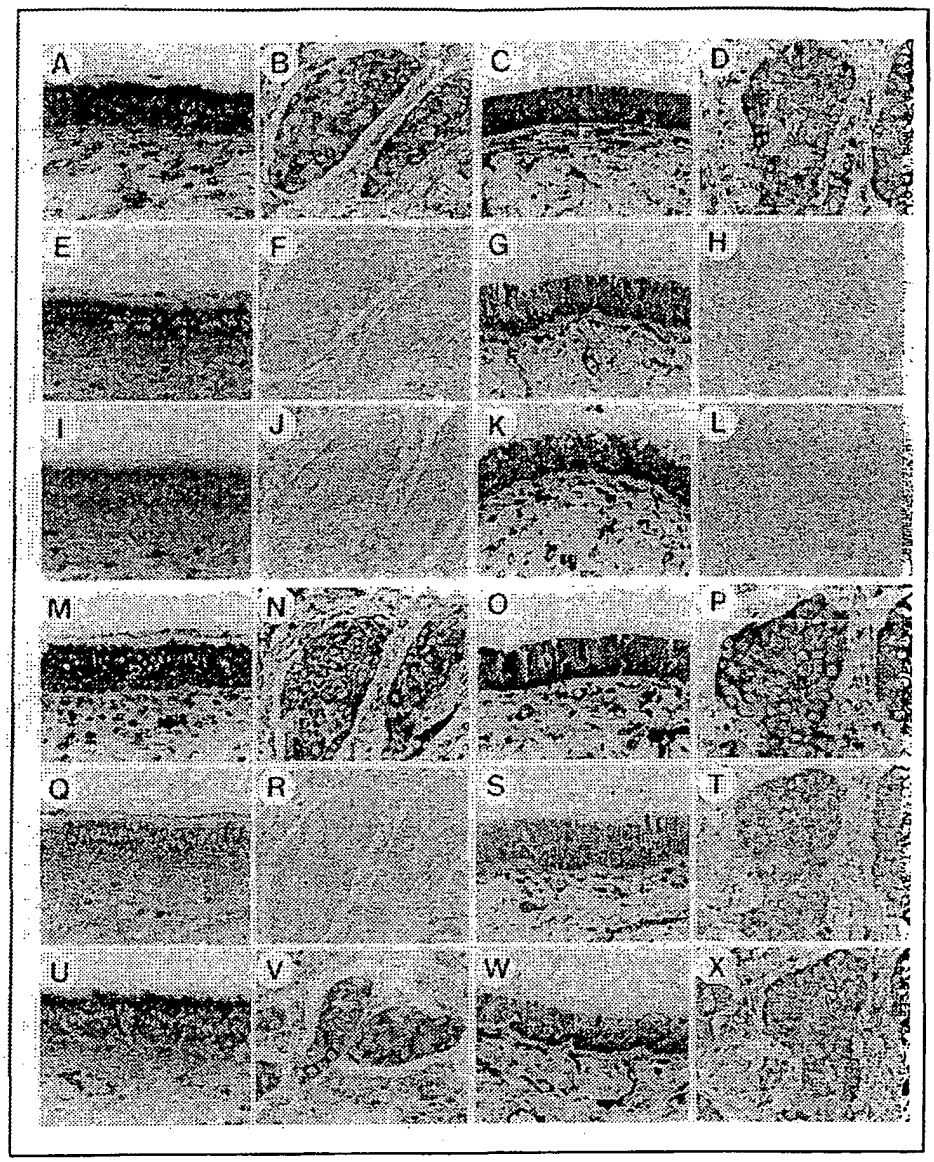



**Fig. 2.** Localization of nuclear retinoic acid receptor (RAR) and retinoid X receptor (RXR) messenger RNAs in matched pairs of either adjacent normal or distant normal bronchial tissue and their corresponding squamous cell carcinomas (SCCs) by in situ hybridization. Consecutive sections of formalin-fixed, paraffin-embedded adjacent normal lung tissue (A, E, I, M, Q, and U) and the corresponding SCCs (B, F, J, N, R, and V) and distant lung tissue (C, G, K, O, S, and W) or the corresponding SCCs (D, H, L, P, T, and X) were hybridized with antisense probes for RARα (A, B, C, and D); RARβ (E, F, G, and H); RARγ (I, J, K, and L); RXRα (M, N, O, and P); RXRβ (Q, R, S, T); or RXRγ (U, V, W, and X).

(mRNAs), whereas only 47% of the corresponding tumors continued to express RARβ. In addition, about 39% of the tumors have lost RARγ expression. The differences in RARβ and RARγ expression between tumor and corresponding distant normal bronchial tissue were statistically significant. The in situ hybridization results of a representative case of SCC are shown in Fig. 2. All of the receptor mRNAs were detected in the distant normal tissue (Fig. 2, C, G, K, O, S, and W), whereas the SCCs failed to express RARβ and RARγ (Fig. 2, H and L, respectively).

### Expression of RARs and RXRs in Paired Specimens of Adjacent Normal Bronchial Lung Tissue and NSCLC From the Same Patient

Table 3 shows that there is a statistically significant decrease in the expression of RARβ, RARγ, and RXRβ in the NSCLC tumor compared with the adjacent normal bronchial tissue, whereas the expression of the other three receptors was similar in the adjacent normal and malignant tissues. Fig. 2 shows representative in situ hybridization results of the analysis of adjacent normal and malignant tissue from the same specimen. All receptors were expressed in the adjacent normal tissue (Fig. 2, A, E, I, M, Q, and U), whereas the SCC failed to express RARβ and RARγ and showed a lower staining of RXRβ and RXRγ (Fig. 2, F, J, R, and V). Because both the adjacent normal and tumor tissue were present in the same histologic section, the comparison of the receptor expression in these tissues is ideal, since the experiment is internally controlled for factors, such as fixation and processing for analysis.

## Discussion

The premise of our investigation is that one of the functions of physiologic retinoids is to prevent carcinogenesis by regulating the differentiation of epithelial tissues. This contention is especially relevant for airway epithelium because vitamin A deficiency results in replacement of mucociliary epithelium with

Table 3. Comparison of expression of RAR and RXR messenger RNAs in matched pairs of distant and adjacent normal bronchial epithelia and the corresponding NSCLC*

| Receptor | % positive cases (No. positive/total) | | | |
|---|---|---|---|---|
| | Distant normal | NSCLC | Adjacent normal | NSCLC |
| RARα | 97.0 (32/33) | 93.9 (31/33) | 100 (40/40) | 95.0 (38/40) |
| RARβ | 91.7 (33/36) | 47.2 (17/36)† | 80.6 (25/31) | 54.8 (17/31)‡ |
| RARγ | 97.0 (32/33) | 90.6 (29/33)§ | 88.9 (24/27) | 66.7 (18/27)‖ |
| RXRα | 100 (24/24) | 100 (24/24) | 100 (29/29) | 100 (29/29) |
| RXRβ | 95.8 (23/24) | 83.3 (20/24) | 92.6 (25/27) | 70.4 (19/27)‖ |
| RXRγ | 90.9 (20/22) | 95.5 (21/22) | 87.5 (14/16) | 93.8 (15/16) |

*See definitions in Table 2.
†$P$ = .0001 between distant normal and NSCLC.
‡$P$ = .021 between adjacent normal and NSCLC.
§$P$ = .0005 between distant normal and NSCLC.
‖$P$ = .034 between adjacent normal and NSCLC.

keratinizing squamous epithelium (squamous metaplasia) and is associated with increased lung cancer incidence (*6*). Because nuclear retinoid receptors mediate most of the effects of retinoids on gene expression, reduced expression of one or more of these receptors may enhance cancer development.

The study reported here is the first to use in situ hybridization for the analysis of the mRNAs for RARs and RXRs in surgical specimens of normal bronchus, normal bronchial lung tissues distant and adjacent to lung tumors, and NSCLC carcinomas. We have demonstrated that RARβ expression is markedly decreased in more than 50% of both adenocarcinomas and SCCs, and that the expression of RARγ and RXRβ was lost in some cases. These findings are similar to our previous demonstration of a decrease in the expression of this receptor in premalignant oral lesions, dysplastic lesions, and head and neck squamous cell carcinomas (*20,21*). However, unlike what was observed in head and neck premalignant and malignant lesions, the lung carcinomas showed suppression of RARγ and RXRβ in some of the cases. Loss of RARβ appears to be the major defect during lung carcinogenesis and this loss did not seem to predispose the tissue to lose also RARγ or vice versa because 17 (22.4%) of 76 of the cases had lost both receptors, 26 (34.2%) of 76 expressed both receptors, 29 (38.2%) of 76 had lost RARβ but retained RARγ expression, and only four (5.3%) of 76 had lost RARγ but not RARβ.

Previous studies (*12-17*) with cultured lung carcinoma cell lines have demonstrated a decreased expression of RARβ. We confirmed that this also occurs in vivo by use of specimens from 79 patients with NSCLC. The former studies employed northern blotting of extracted RNA for assessment of RARβ expression. Thus, Gebert et al. (*12*) found decreased RARβ expression in at least 50% of 33 lung cancer cell lines and in 30% of nine lung cancer specimens.

Although decreased expression of RARβ appears to be a common event in lung carcinogenesis, the underlying molecular mechanism is unclear. The RARβ gene promoter includes a RARE, which can be activated by RXR–RAR heterodimers. However, many lung cancer cell lines exhibit defects in transcription of RARβ–RARE, possibly because of inactivation or absence of *trans*-acting co-factors that are required for the transcription (*16,17*). The RARγ gene promoter also has a RARE, and RXRβ has been shown to be regulated by retinoids in embryonal carcinoma cells, so their decreased expression in some of the lung cancer cases may also reflect defects in transcription activation.

The involvement of retinoid receptors in the regulation of gene expression in tracheal epithelial cells (*22*) and in suppression of growth of transformed tracheal epithelial cells (*23*) has been demonstrated. Recent evidence implicated RARβ specifically in tumor suppression in vivo; transfection of a human epidermoid lung cancer in vitro with an RARβ expression vector resulted in decreased tumorigenicity in nude mice (*18*). In addition, transgenic mice expressing antisense RARβ2 developed carcinomas 14-18 months after birth (*24*). We have recently reported that blocking the expression of RARγ in head and neck SCC cells resulted in resistance to retinoid-induced growth inhibition and a loss of the ability of RA to exert anti-AP-1 activity (*25*). Thus, the loss of expression of either RARβ or RARγ in lung tissue could enhance cancer development. Future studies will be directed toward identifying the genes that are regulated specifically by RARβ to gain a better understanding of the pathway by which this receptor may suppress carcinogenesis.

## References


(*1*) Parker SL, Tong T, Bolden S, Wingo PA. Cancer Statistics, 1997. CA Cancer J Clin 1997;47:5-27.

(*2*) Sporn MB, Newton DL. Chemoprevention of cancer with retinoids. Fed Proc 1979;38:2528-34.

(*3*) Jetten AM, Nervi C, Vollberg TM. Control of squamous differentiation in tracheobronchial and epidermal epithelial cells: role of retinoids. Natl Cancer Inst Monog 1992;13:93-100.

(*4*) Kelloff GJ, Boone CW, Steele VK, Perloff M, Crowell J, Doody LA. Development of chemopreventive agents for lung and upper aerodigestive tract cancers. J Cell Biochem Suppl 1993;17F:2-17.

(*5*) Moon RC, Mehta RG, Rao KJ. Retinoids and cancer in experimental animals. In: Sporn MB, Roberts AB, Goodman DS, editors. The retinoids. New York: Raven Press, 1994:573-95.

(*6*) Hong WK, Itri LM. Retinoids and human cancer. In: Sporn MB, Roberts AB, Goodman DS, editors. The retinoids. New York: Raven Press, 1994: 597-658.

(*7*) Hong WK, Lippman SM, Hittelman WN, Lotan R. Retinoid chemoprevention of aerodigestive cancer: from basic research to the clinic. Clin Cancer Res 1995;1:677-86.

(*8*) Pastorino U, Infante M, Maioli M, Chiesa G, Buyse M, Firket P, et al. Adjuvant treatment of stage I lung cancer with high-dose vitamin A. J Clin Oncol 1993;11:1216-22.

(*9*) Chambon P. The retinoid signaling pathway: molecular and genetic analyses. Semin Cell Biol 1994;5:115-25.

(*10*) Mangelsdorf DJ, Umesono K, Evans RM. The retinoid receptors. In: Sporn

MB, Roberts AB, Goodman DS, editors. The retinoids. New York: Raven Press, 1994:319-49.
(11) Pfahl M. Vertebrate receptors: molecular biology, dimerization and response elements. Semin Cell Biol 1994;5:95-103.
(12) Gebert JF, Moghal N, Frangioni JV, Sugarbaker DJ, Neel BG. High frequency of retinoic acid receptor beta abnormalities in human lung cancer [published erratum appears in Oncogene 1992;7:821]. Oncogene 1991;6:1859-68.
(13) Houle B, Leduc F, Bradley WE. Implication of RARB in epidermoid (squamous) lung cancer. Genes Chromosomes Cancer 1991;3:358-66.
(14) Nervi C, Vollberg TM, George MD, Zelent A, Chambon P, Jetten AM. Expression of nuclear retinoic acid receptors in normal tracheobronchial cells and in lung carcinoma cells. Exp Cell Res 1991;195:163-70.
(15) Geradts J, Chen JY, Russell EK, Yankaskas JR, Nieves L, Minna J. Human lung cancer cell lines exhibit resistance to retinoic acid treatment. Cell Growth Differ 1993;4:799-809.
(16) Zhang XK, Liu Y, Lee MO, Pfahl M. A specific defect in the retinoic acid response associated with human lung cancer cell lines. Cancer Res 1994;54:5663-9.
(17) Moghal N, Neel BG. Evidence for impaired retinoic acid receptor-thyroid hormone receptor AF-2 cofactor activity in human lung cancer. Mol Cell Biol 1995;15:3945-59.
(18) Houle B, Rochette-Egly C, Bradley WE. Tumor-suppressive effect of the retinoic acid receptor beta in human epidermoid lung cancer cells. Proc Natl Acad Sci U S A 1993;90:985-9.
(19) Xu XC, Clifford JL, Hong WK, Lotan R. Detection of nuclear retinoic acid receptor mRNA in histological tissue sections using nonradioactive in situ hybridization histochemistry. Diag Mol Pathol 1994;3:122-31.
(20) Xu XC, Ro JY, Lee JS, Shin DM, Hong WK, Lotan R. Differential expression of nuclear retinoic acid receptors in normal, premalignant, and malignant head and neck tissues. Cancer Res 1994;54:3580-7.
(21) Lotan R, Xu XC, Lippman SM, Ro JY, Lee JS, Lee JJ, et al. Suppression of retinoic acid receptor-beta in oral premalignant lesions and its up-regulation by isotretinoin. N Engl J Med 1995;332:1405-10.
(22) Denning MF, Verma AK. Involvement of retinoic acid nuclear receptors in retinoic acid-induced tissue transglutaminase gene expression in rat tracheal 2C5 cells. Biochem Biophys Res Commun 1991;175:344-50.
(23) Ahn MJ, Langenfeld J, Moasser MM, Rusch V, Dmitrovsky E. Growth suppression of transformed human bronchial epithelial cells by all-*trans*-retinoic acid occurs through specific retinoid receptors. Oncogene 1995;11:2357-64.
(24) Berard J, Laboune F, Mukuna M, Masse S, Kothary R, Bradley WE. Lung tumors in mice expressing an antisense RARbeta2 transgene. FASEB J 1996;10:1091-7.
(25) Oridate N, Esumi N, Lotan D, Hong WK, Rochette-Egly C, Chambon P, et al. Implication of retinoic acid receptor gamma in squamous differentiation and response to retinoic acid in head and neck SqCC/Y1 squamous carcinoma cells. Oncogene 1996;12:2019-28.

## Notes

Supported by Public Health Service grant CA68437 from the National Cancer Institute, National Institutes of Health, Department of Health and Human Services, by a grant from the Lamar Fleming and O. H. Davenport Fund awarded by the Texas Medical Center, Houston, and by grants from the Associazione Italiana Ricerca Cancro and Consiglio Nazionale delle Ricerce Special Project ARCRO.

We thank Susan Cweren for preparing some of the tissue sections, Ren Yu for assistance in statistical analysis, and Dr. H. A. Pierotti for support and critical discussions.

Manuscript received November 11, 1996; revised February 25, 1997; accepted February 27, 1997.

09/919195

| | |
|---|---|
| ACCESSION NUMBER: | 129:258278 CA |
| TITLE: | Retinoic acid-receptor activation of SP-B gene transcription in respiratory epithelial cells |
| AUTHOR(S): | Yan, Cong; Ghaffari, Manely; Whitsett, Jeffrey A.; Zeng, Xin; Sever, Zvjezdana; Lin, Sui |
| CORPORATE SOURCE: | Division of Pulmonary Biology, Children's Hospital Medical Center, Cincinnati, OH, 45229-3039, USA |
| SOURCE: | American Journal of Physiology (**1998**), **275**(2, Pt. 1), **L239**-L246<br>CODEN: AJPHAP; ISSN: 0002-9513 |
| PUBLISHER: | American Physiological Society |
| DOCUMENT TYPE: | Journal |
| LANGUAGE: | English |
| REFERENCE COUNT: | 42 THERE ARE 42 CITED REFERENCES AVAILABLE FOR THIS RECORD. ALL CITATIONS AVAILABLE IN THE RE FORMAT |

# Retinoic acid-receptor activation of SP-B gene transcription in respiratory epithelial cells

CONG YAN, MANELY GHAFFARI, JEFFREY A. WHITSETT, XIN ZENG, ZVJEZDANA SEVER, AND SUI LIN
*Division of Pulmonary Biology, Children's Hospital Medical Center, Cincinnati, Ohio 45229-3039*

**Yan, Cong, Manely Ghaffari, Jeffrey A. Whitsett, Xin Zeng, Zvjezdana Sever, and Sui Lin.** Retinoic acid-receptor activation of SP-B gene transcription in respiratory epithelial cells. *Am. J. Physiol.* 275 (*Lung Cell. Mol. Physiol.* 19): L239–L246, 1998.—Retinoids are known to play important roles in organ development of the lung. Retinoids exert their activity by modulating the expression of numerous genes, generally influencing gene transcription, in target cells. In the present work, the mechanism by which retinoic acid (RA) regulates surfactant protein (SP) B expression was assessed in vitro. RA (9-*cis*-RA) enhanced SP-B mRNA in pulmonary adenocarcinoma cells (H441 cells) and increased transcriptional activity of the SP-B promoter in both H441 and mouse lung epithelial cells (MLE-15). Cotransfection of H441 cells with retinoid nuclear receptor (RAR)-α, -β, and -γ and retinoid X receptor (RXR)-γ further increased the response of the SP-B promoter to RA. Treatment of H441 cells with RA increased immunostaining for the SP-B proprotein and increased the number of cells in which the SP-B proprotein was detected. An RA responsive element mediating RA stimulation of the human SP-B promoter was identified. RAR-α and -γ and RXR-α but not RAR-β or RXR-β and -γ were detected by immunohistochemical analysis of H441 cells. RA, by activating RAR activity, stimulated the transcription and synthesis of SP-B in pulmonary adenocarcinoma cells.

surfactant protein B; glucocorticoid receptor; thyroid transcription factor-1

RETINOIC ACID (RA), a derivative of vitamin A, plays highly diverse roles in cell proliferation, differentiation, and organ development. RA exerts its biological activity by binding to retinoid nuclear receptors (RARs) and retinoid X receptors (RXRs) in the nucleus of target cells. Ligand-dependent RARs and RXRs form heterodimers that bind to RA response elements (RAREs) on many genes, thereby modulating transcriptional activity. Both RARs and RXRs belong to the superfamily of nuclear receptors. RARs are activated by all *trans*-RA and 9-*cis*-RA, whereas RXRs are only activated by 9-*cis*-RA. Three isotypes of RAR, α, β and γ, encoded by distinct genes (for reviews, see Refs. 15, 19) have been identified. Retinoid receptors consist of a DNA-binding domain that contains $Zn^{2+}$ finger motifs, a ligand-binding domain, a ligand-independent transcriptional activation domain, a ligand-dependent transcriptional activation domain, a dimerization domain, and an F region of unknown function (for a review, see Ref. 18). Through these various domains, RARs interact with other transcriptional and signaling factors, including CBP/p300 (14), activator protein-1 (32), TFIIH (31), and $TAF_{II}135$ (24).

Retinoids have pleiotropic effects in many target organs including the lung. Vitamin A deficiency is associated with squamous metaplasia of the respiratory epithelium. Clinical studies in premature infants demonstrated a correlation between low serum levels of vitamin A and chronic lung disease after respiratory distress syndrome (33). A previous study (23) demonstrated that RA influences fetal lung morphogenesis and differentiation. All three isotypes of RAR mRNAs are expressed in pulmonary tissues during fetal development (22). In situ hybridization analysis showed that RAR-β was expressed in the epithelium of proximal bronchi in *day 14.5* postconception embryos, whereas RAR-α and -γ mRNAs were expressed rather weakly and homogeneously in developing lung tissue (9). RA treatment of both human and rat fetal lung explants in culture altered surfactant protein (SP) and mRNA concentrations (3, 6, 11, 25). In human lung explant, RA reduced SP-A and SP-C mRNA levels. In contrast, all *trans*-RA increased SP-B mRNA levels in a concentration-dependent manner, with the maximum increase observed at 3 μM (25). In vitamin A-deficient animals, fetal lung weight was significantly decreased in association with decreased phosphatidylcholine content, a marker of respiratory epithelial cell differentiation (22). Mice bearing null mutations in both RAR-α and RAR-β displayed some of the organ defects, including hypoplastic lungs (23), providing further support for the role of RARs in lung morphogenesis.

Pulmonary surfactant is a complex mixture of lipids and proteins that reduces surface tension at the air-liquid interface in the alveoli. Surfactant lipids are synthesized primarily by alveolar type II epithelial cells and are stored in lamellar bodies that are secreted into the air space. SP-A, SP-B, SP-C, and SP-D are also synthesized primarily by type II or bronchiolar epithelial cells and play critical roles in maintaining stability of the surfactant layer (SP-B and SP-C) and in host defense (SP-A and SP-D). The mechanisms by which RA and its RARs/RXRs influence surfactant homeostasis in type II epithelial cells in the respiratory epithelium, especially at the level of gene transcription, are largely unknown.

SP-B is a 79-amino acid amphipathic peptide produced by the proteolytic cleavage of SP-B proprotein (proSP-B) by type II epithelial cells. The SP-B peptide is stored in lamellar bodies and secreted with phospholipids into the airway lumen (for a review, see Ref. 36). SP-B is a critical component of the surfactant complex and is essential for the formation of tubular myelin and the stability and rapid spreading of surfactant phospholipids (36). Genetic defects in SP-B cause respiratory

failure after birth in both humans and SP-B gene-targeted mice (7, 26, 27).

SP-B homeostasis is modulated at multiple levels. SP-B gene transcription is influenced by thyroid transcription factor-1 (TTF-1) and hepatocyte nuclear factor-3 (HNF-3) (4, 40). SP-B gene transcription is further enhanced by cAMP and protein kinase A-dependent phosphorylation of TTF-1 (42). Glucocorticoids stimulate SP-B gene expression in both cell lines and lung explants (1, 2, 10, 34). Phorbol ester strongly inhibited SP-B gene expression (30, 35). At the posttranscriptional level, SP-B mRNA stability is enhanced by glucocorticoids and decreased by tumor necrosis factor-α and phorbol ester (28, 29, 38).

In the present study, both all *trans*- and 9-*cis*-RA enhanced transcription of the human and mouse SP-B promoters in human pulmonary adenocarcinoma cells (H441) and mouse lung epithelial cells (MLE-15). The H441 cell line was isolated from a human lung adenocarcinoma. The MLE-15 cell line was derived from mouse lung tumor cells immortalized by the SV40 large T antigen in vivo (37). Both cell lines have been used extensively to characterize SP-A, SP-B, and SP-C gene transcription.

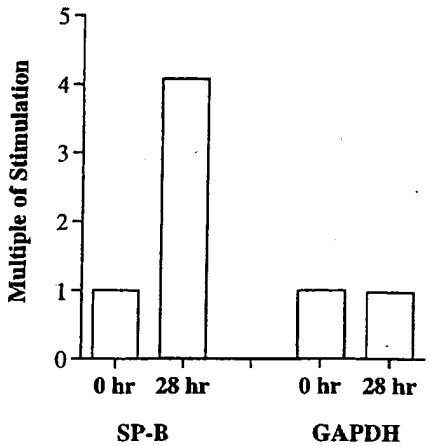



Fig. 2. RA increases endogenous hSP-B mRNA. Total RNAs from H441 cells treated with 9-*cis*-RA for 0 and 28 h were prepared and reverse transcribed. Reverse-transcribed cDNA products were amplified by PCR with a primer pair for hSP-B gene and a primer pair for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene as described in MATERIALS AND METHODS. GAPDH was used as a control. PCR product intensity of RA treatment at 0 h was defined as 1. Values are means; $n$ = 2 experiments; each RT-PCR determination was performed in triplicate.

## MATERIALS AND METHODS

*Cell culture.* H441 cells were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum, glutamine, and penicillin-streptomycin. Cells were maintained at 37°C in 5% $CO_2$-air and passaged weekly. The murine clonal respiratory epithelial cell line MLE-15 was propagated in HITES medium (37) containing 4% fetal bovine serum and maintained as above.

*Plasmid constructs.* The region from +41 to −2240 of the human SP-B (hSP-B) promoter was generated by PCR with synthetic oligonucleotide primers with the pΔ5′-2240 SP-B chloramphenicol acetyltransferase (CAT) construct as a template as described previously (40). The upstream primer with the *Mlu* I site was 5′-CGCACGCGTACCTGCAGGTCAACG-GATCA-3′. The downstream primer with the *Xho* I site was 5′-GCGCTCGAGCCACTGCAGCAGGTGTGACTC. The PCR products were digested with *Mlu* I and *Xho* I restriction enzymes and ligated with *Mlu* I-*Xho* I-digested pGL2-B luciferase reporter plasmids (Promega). The correctness of the hSP-B 2240 promotor fragment (hSP-B-2240) luciferase reporter construct was confirmed by DNA sequencing. The murine (m) SP-B promoter fragment −1797/+42 (mSP-B-1797) was subcloned in the pBLCAT6 reporter vector as described previously (5). The human TTF-1 1.7-kb luciferase reporter gene was made previously (13). Human RAR and RXR expression vectors hRAR-α/pSG5, hRAR-β/pSG5, hRAR-γ1/pSG5, and hRXR-γ/pSG5 were kindly provided by Dr. Pierre Chambon. Human glucocorticoid-receptor (GR) expression vector pR5-hGR-α was kindly provided by Dr. Ronald M. Evans.

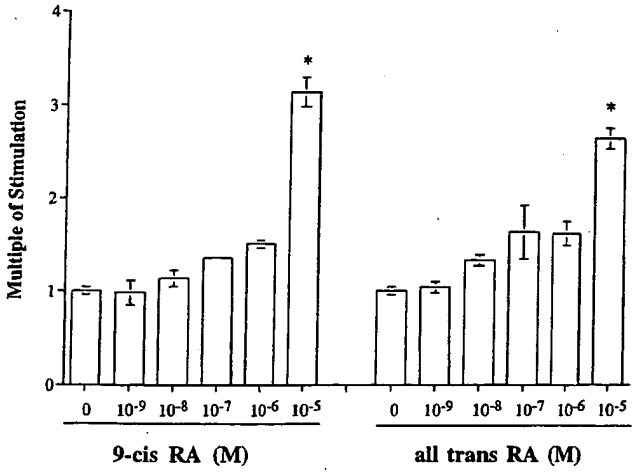



Fig. 1. Retinoic acid (RA) stimulates human surfactant protein (hSP) B 2240 promoter (hSP-B-2240) activity. Human pulmonary adenocarcinoma (H441) cells were transfected with hSP-B-2240 luciferase reporter vector and treated with all *trans*- and 9-*cis*-RA. Luciferase activity was measured 48 h after treatment. Activity without RA treatment was defined as 1. Activities were measured in light units of optical density (OD) of β-galactosidase. Values are means ± SD; $n$ = 3 experiments, each performed in triplicate. ANOVA showed significant stimulatory effect of 9-*cis*-RA on hSP-B promoter. *Significant difference from control values, $P < 0.02$.

*Transfection, luciferase, and CAT assays.* To determine the effects of all *trans*-RA and 9-*cis*-RA on the hSP-B promoter luciferase reporter constructs, transient transfection and luciferase assays were performed as previously described (40, 41) with minor modification. Briefly, H441 cells were seeded at a density of $2 \times 10^5$ cells/well in 6-well plates. The hSP-B reporter constructs (0.5 μg) were transfected into H441 cells by lipofectin transfection (GIBCO BRL). In each transfection, 0.5 μg of pCMV-β-gal plasmid was included for normalization of transfection efficiency. For quantification of β-galactosidase activity, one unit of optical density of β-galactosidase in the protein extract was defined as hydrolysis of *o*-nitrophenyl-β-D-galactopyranoside that generates absorbance to 1 optical density unit at 420 nm at 37°C. After 2 days of incubation with various concentrations of all *trans*- and 9-*cis*-RA, the cells were lysed and luciferase activity assays were performed with the luciferase assay system (Promega). The light units were assayed by luminometry (monolight 2010, Analytical Luminescence Laboratory, San Diego, CA). In RAR/RXR cotransfection assays, 0.5 μg of various RAR and RXR con-

structs was cotransfected with 0.5 μg of the hSP-B reporter constructs and treated with 10 μM 9-*cis*-RA. Each experiment was repeated at least three times. Human TTF-1 promoter studies with the luciferase reporter gene with RARs/RXR-γ were performed as outlined above. No effect of RA and RARs/RXR-γ on expression of β-galactosidase activity was observed in transfection assays in H441 cells.

Transient transfection study of the mSP-B-1797 was performed with the calcium precipitation method. A mixture of mSP-B-1797 (0.67 pmol/well) and pCMB-β-gal (1.25 μg/well) was used for transfection followed by the addition of 10 μM 9-*cis*-RA for 2 days. Cell extracts were prepared with three freeze-thaw cycles, and the pellets were resuspended in 50–100 μl of 0.25 M Tris, pH 7.8. CAT assays were performed as previously described (5, 41). Chromatograms of [$^{14}$C]chloramphenicol and its acetylated derivatives were quantitated with a Molecular Dynamics phosphorimager (Storm 680).

*RT-PCR.* Total RNA was purified from H441 cells treated with 10 μM 9-*cis*-RA for 0 and 28 h following the procedures described previously (39). The quality of RNA samples was assessed on 1% agarose gel after ethidium bromide staining. Thirty micrograms of total RNA were reverse transcribed with the oligo(dT) primer (NEN) by superscript RT enzyme (GIBCO BRL) in the presence of deoxynucleoside triphosphate and first-strand buffer (GIBCO BRL). Five micrograms of reverse transcripts were amplified in 30 cycles of PCR with a SP-B primer pair corresponding to exons 8 and 10 (upstream primer, 5′-GGTCGCCGACAGGAGAATGGCTGC-3′; downstream primer, 5′-AAGGTCGGGGCTGTGGATACACTG-3′). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a control, and the same reverse-transcribed cDNAs were used for PCR with a GAPDH primer pair (upstream primer, 5′-CAGAAGACTGTGGATGGCCCC-3′; downstream primer, 5′-GTCCACCACCCTGTTGCTGTAGCC-3′). Five micrograms of reaction products were separated on 1% agarose gel before being stained. Intensities of the PCR product bands were quantitatively analyzed by an IS-1000 Digital Imaging System (Alpha Innotech, San Leandro, CA).

*Immunohistochemistry.* H441 or HeLa cells were seeded onto Permanox chamber slides (Fisher) at densities ranging from $10^4$ to $10^5$ cells/chamber (2 chambers/slide) as described previously (12). For immunostaining of SP-B, RAR, and RXR proteins, slides were pretreated with 0.1 M PBS containing Triton X-100, pH 7.4, plus 5% goat serum for 2 h at room temperature before incubation with a 1:500 dilution of RAR and RXR rabbit polyclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) or a 1:100 dilution of proSP-B polyclonal antibodies overnight. The next day, the slides were washed five times in 0.1 M PBS with Triton X-100 solution, and biotinylated goat anti-rabbit IgG was added to the serum blocking solution (45 μl IgG/10 ml) for 30 min, followed by five washes in 0.1 M PBS with Triton X-100 solution. The slides were then treated with avidin-biotin reagent according to the directions in the Vectastain Elite Kit (Vector Laboratories, Burlingame, CA). The slides were visualized by a Nikon Microphot-FXA video system. For transfection studies, 2 μg of the RAR-β expression plasmid were transfected into cells with the lipofectin transfection kit from GIBCO BRL as previously described (12). Immunohistochemical staining of the transfected cells was then performed as described above.

*Electrophoretic mobility shift assay.* An oligonucleotide corresponding to the hSP-B promoter −415 to −440 was synthesized, annealed, and purified. The oligonucleotide was radiolabeled by [γ-$^{32}$P]ATP and kinase and incubated with 100 ng of the purified RAR-γ-glutathione *S*-transferase (GST) fusion protein as suggested by the manufacturer (Santa Cruz Biotechnology). In the absence of RXRs, a relatively higher concentration of RAR is required due to its low DNA-binding affinity. Antibody-recognizing RAR-γ (1 μg) was used for supershift assay. An electrophoretic mobility shift assay (EMSA) was performed by following the procedures previously described (40).

## RESULTS

*Dose-dependent stimulation of hSP-B promoter activity by all trans- and 9-cis-RA.* To test the effects of RA on SP-B gene transcription, the hSP-B-2240 luciferase reporter construct was transfected into H441 cells. Cells were treated with all *trans*- or 9-*cis*-RA ($10^{-5}$ to $10^{-9}$ M). Both all *trans*- and 9-*cis*-RA enhanced hSP-B-2240 activity (Fig. 1). Activation was observed at $10^{-8}$ M of both 9-*cis*- and all *trans*-RA (~10% increase). Significant activation was observed at $10^{-5}$ M as assessed by one-way ANOVA ($P < 0.02$).

*RA induced endogenous hSP-B mRNA in H441 cells.* Because the transcriptional activity of the hSP-B pro-

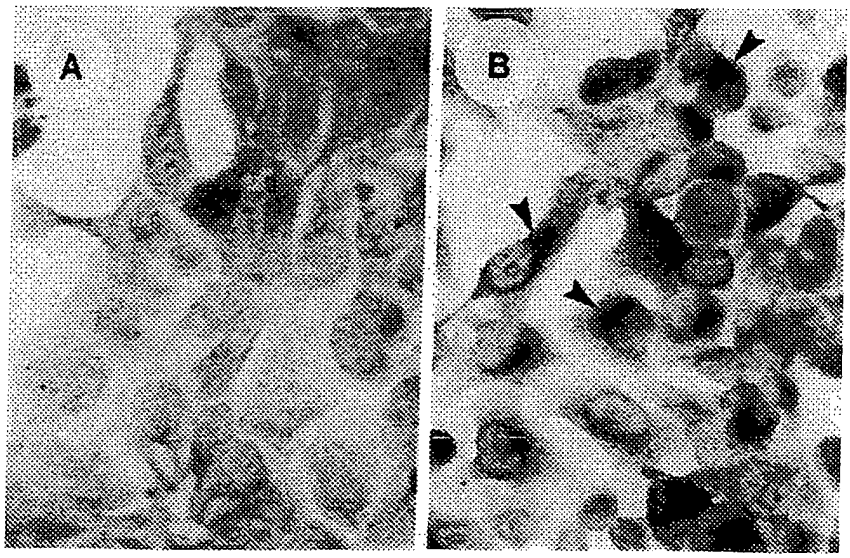



Fig. 3. Stimulation of SP-B proprotein (proSP-B; arrowheads) synthesis by RA. H441 cells were treated without (*A*) and with (*B*) $10^{-5}$ M 9-*cis*-RA for 2 days. Cells were immunostained with rabbit proSP-B polyclonal antibody as described in MATERIALS AND METHODS.

Table 1. *Transcriptional stimulation of the hSP-B 2.24-kb promoter by RAR/RXR and inhibition by GR in H441 cells*

| Nuclear Receptor Plasmid | | Multiple of Stimulation |
|---|---|---|
| | *Study 1* | |
| None | −9-*cis*-RA | 1.00 ± 0.06 |
| | +9-*cis*-RA | 3.38 ± 0.22* |
| RAR-α/RXR-γ | +9-*cis*-RA | 6.89 ± 0.17* |
| RAR-β/RXR-γ | +9-*cis*-RA | 8.13 ± 0.15* |
| RAR-γ/RXR-γ | +9-*cis*-RA | 5.42 ± 0.27* |
| | *Study 2* | |
| None | −Dex | 1.00 ± 0.20 |
| | +Dex | 0.75 ± 0.15 |
| GR | −Dex | 0.65 ± 0.46 |
| | +Dex | 0.37 ± 0.045* |

Values are means ± SD; $n$ = 3 experiments, each performed in triplicate. Human pulmonary adenocarcinoma (H441) cells were cotransfected with human surfactant protein (hSP) B 2.4-kb luciferase reporter vector and retinoic acid (RA)-receptor/retinoid X-receptor (RAR/RXR) or glucocorticoid-receptor (GR) expression plasmids. Transfected cells were treated with 10 μM 9-*cis*-RA or 50 nM dexamethasone (Dex). Luciferase activity was measured 48 h later. Activity of hSP-B 2.4-kb luciferase reporter vector without 9-*cis*-RA treatment in absence of RAR/RXR cotransfection was defined as 1 for multiple of stimulation. Activity is measured in light units of optical density of β-galactosidase. *Significant stimulatory effect of 9-*cis*-RA and RARs/RXR-γ and significant inhibitory effect of Dex and GR on hSP-B 2.24-kb promoter, $P < 0.02$ by ANOVA.

moter was stimulated by RA, the effects of RA on the expression of endogenous hSP-B mRNA were assessed by RT-PCR (Fig. 2). H441 cells were treated with 9-*cis*-RA ($10^{-5}$ M) for 0 and 28 h. Total RNAs were extracted from cells, reverse transcribed, and amplified by PCR with an hSP-B-specific primer pair, with a GAPDH-specific primer pair as a control. After 28 h of exposure to 9-*cis*-RA, SP-B mRNA was significantly increased ($P < 0.02$ by paired $t$-test). No significant stimulation was observed in GAPDH mRNA ($P > 0.15$). The H441 cells were also treated with dexamethasone (50 nM). SP-B mRNA increased 30- to 50-fold after 28 h of treatment (data not shown), consistent with previous observations (28).

*Increased proSP-B staining after treatment with 9-cis-RA.* H441 cells were treated for 48 h with 9-*cis*-RA ($10^{-5}$ M) and immunohistochemically stained with anti-human proSP-B polyclonal antibody. Intracellular proSP-B staining of H441 cells was increased by 9-*cis*-RA (Fig. 3).

*RAR/RXR activation of the hSP-B promoter.* To assess the effects of RAR on hSP-B promoter activity, three forms of human RAR (a, β, and γ)-RXR-γ expression plasmids were cotransfected with the hSP-B-2240 luciferase reporter construct into H441 cells. Cotransfection of RAR-α/RXR-γ, RAR-β/RXR-γ, and RAR-γ/RXR-γ into H441 cells further increased hSP-B 2.24-kb promoter activity, resulting in a seven- to ninefold induction after treatment with 9-*cis*-RA ($P < 0.05$ by one-way ANOVA; Table 1).

Glucocorticoids are known to enhance SP-B mRNA in vivo and in H441 cells. To determine the potential role of the GR in SP-B gene regulation in H441 cells, a GR expression plasmid was cotransfected with the hSP-B-2240 luciferase reporter construct (Table 1). In the absence of a GR, dexamethasone (50 nM) slightly decreased the activity of the hSP-B-2240 luciferase reporter gene. Cotransfection with a GR also slightly decreased the activity of the hSP-B-2240 luciferase reporter gene in the absence of dexamethasone. Surprisingly, cotransfection with a GR in the presence of dexamethasone significantly repressed luciferase activity of the hSP-B-2240 from 100 ± 20 to 37 ± 4.5% of luciferase activity ($P < 0.02$ by one-way ANOVA).

*Identification of RAREs on the hSP-B promoter.* In the region of −500 to −218 of the hSP-B promoter, a sequence (−440 to −415) resembling a direct repetition of RARE (core motif, A/GGG/TTCA) was identified (Fig. 4*A*).

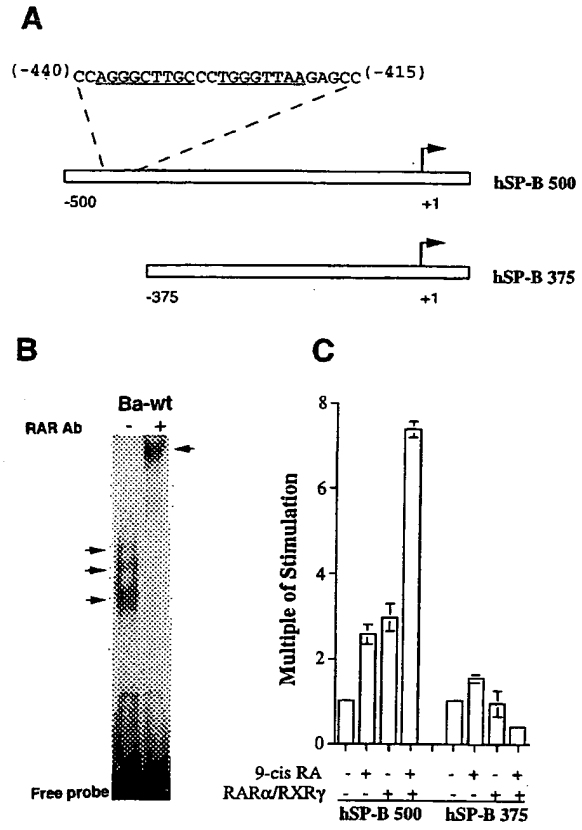



Fig. 4. Identification of RA response element (RARE) on hSP-B promoters. *A*: schematic illustration of hSP-B-500, hSP-B-375, and nucleotide sequence (Ba-wt) from −415 to −440. Underlined sequences resemble direct repetition of RARE. *B*: electrophoretic mobility shift assay of purified RA receptor (RAR)-γ-glutathione *S*-transferase protein with a nucleotide oligomer corresponding to −415 to −440 of hSP-B promoter. Antibody (Ab) recognizing RAR-γ was included to supershift DNA-protein complexes. Arrows indicate DNA-protein complexes. *C*: H441 cells were transfected with hSP-B-500 or hSP-B-375 luciferase reporter vector, treated with 9-*cis*-RA ($10^{-5}$ M), and cotransfected with RAR-α/retinoid X receptor (RXR)-γ. +, Presence; −, absence. Luciferase activity was measured 48 h after treatment. Activity without RA treatment was defined as 1. Activities were measured in light units of OD of β-galactosidase. Values are means ± SD; $n$ = 3 experiments, each performed in triplicate. ANOVA showed significant stimulatory effect of 9-*cis*-RA and RAR-α/RXR-γ on hSP-B-500, $P < 0.02$.

This oligonucleotide, Ba-wt (−415 to −440), was synthesized and incubated with the purified RAR-γ-GST fusion protein. Interestingly, three specific RARE-RAR-γ complexes were detected by EMSA, and an antibody recognizing RAR-γ supershifted all three complexes (Fig. 4*B*). In the promoter deletion studies, activity of a hSP-B promoter (hSP-B-500) containing this RARE was significantly enhanced by cotransfection with RAR-α/RXR-γ and 9-*cis*-RA treatment as assessed by luciferase reporter assay and one-way ANOVA ($P < 0.02$). A promoter construct (hSP-B-375) lacking the RARE sequence was completely unresponsive to RAR-α/RXR-γ and 9-*cis*-RA stimulation (Fig. 4*C*).

*Immunohistochemical detection of RAR and RXR proteins in H441 and HeLa cells.* Immunohistochemical staining of H441 cells was performed with distinct antibodies recognizing the α, β, and γ isotypes of RAR and RXR. RAR-α and -γ and RXR-α were detected in the nuclei of H441 cells (Fig. 5*A*), whereas RAR-β and RXR-β and -γ were not detected. In contrast, HeLa cells were stained by antibodies recognizing RAR-α and -γ and all three forms of the RXR isotype (Fig. 5*B*). In both HeLa and H441 cells, RAR and RXR staining was stronger in the nuclei than in the cytoplasm. Although RAR-β was not detected in either H441 or HeLa cells, RAR-β was readily detected after transfection with the hRAR-β expression plasmid in both cell lines.

*9-cis-RA stimulates the activity of the mSP-B promoter in MLE-15 cells.* The effect of RA on the reporter construct consisting of the mSP-B-1797 (5) was tested in MLE-15 cells. 9-*cis*-RA ($10^{-5}$ M) activated the mSP-B-CAT 1797-bp reporter construct to a similar extent as the hSP-B 2240 construct ($P < 0.02$ by paired *t*-test; Fig. 6).

*9-cis-RA does not alter hTTF-1 promoter activity in H441 cells.* SP-B gene transcription is strongly influ-

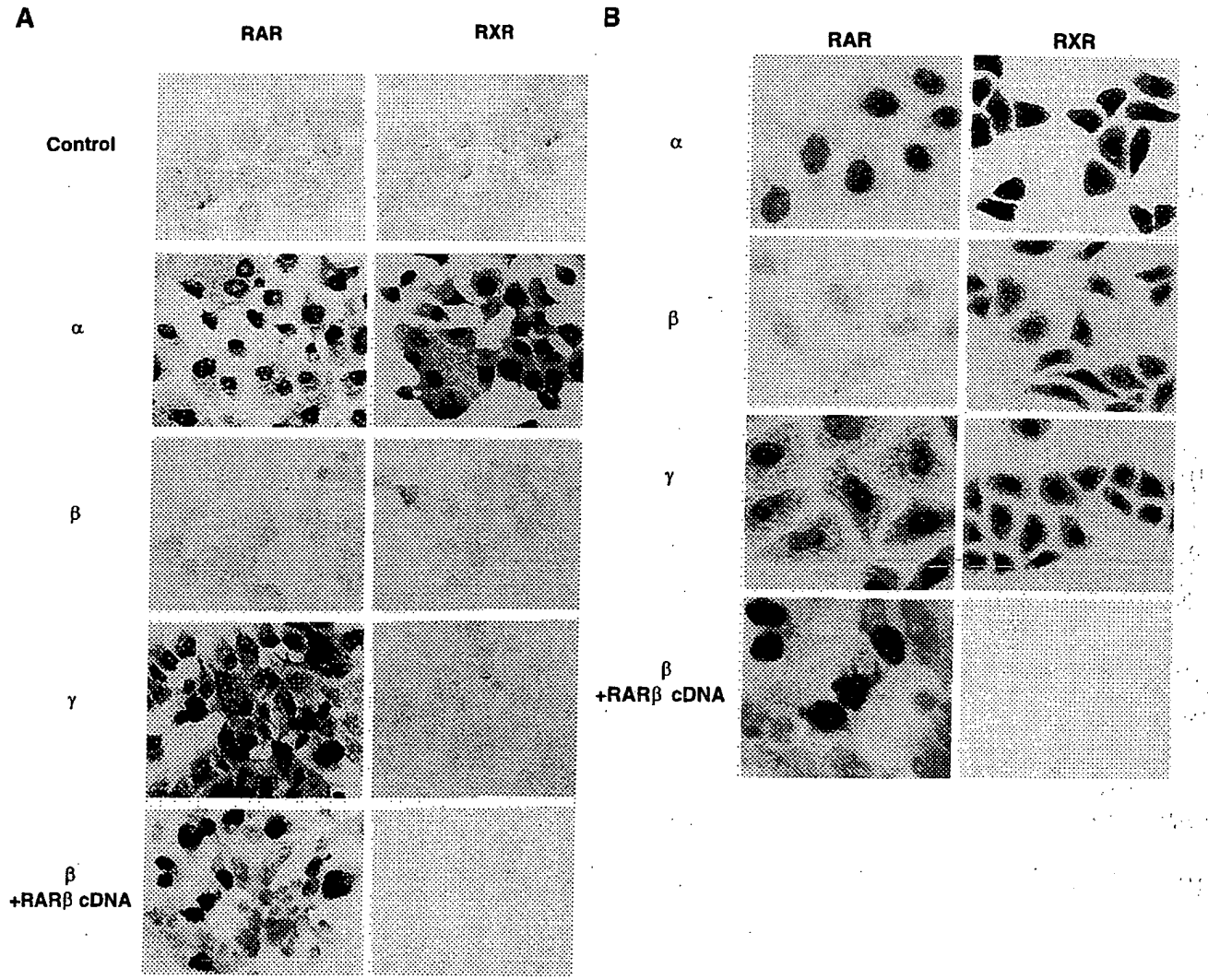



Fig. 5. Immunohistochemical staining of RARs and RXRs in H441 (*A*) and HeLa (*B*) cells. H441 and HeLa cells were immunostained with rabbit polyclonal antibodies against RAR-α, -β, and -γ and RXR-α, -β, and -γ. As a positive control for RAR-β protein, H441 and HeLa cells were transfected with RAR-β expression vector for 2 days and immunostained with rabbit RAR-β polyclonal antibody.

enced by TTF-1. Previously, immunohistochemical and in situ hybridization studies (13, 17) demonstrated that TTF-1 expression was colocalized with SP-B in both human and mouse lungs. TTF-1 is also expressed in H441 and MLE-15 cells where it regulates SP-B gene transcription. To test the possibility that the effects of RA on SP-B expression were mediated by changes in TTF-1 gene transcription, the effect of RA on the activity of the human TTF-1 promoter region 1–1,700 bp was assessed. Cotransfection of RAR/RXR and GR had no effect on the hTTF-1 1.7-kb construct in the presence or absence of RA and dexamethasone ($P > 0.1$ by paired $t$-test; Fig. 7).

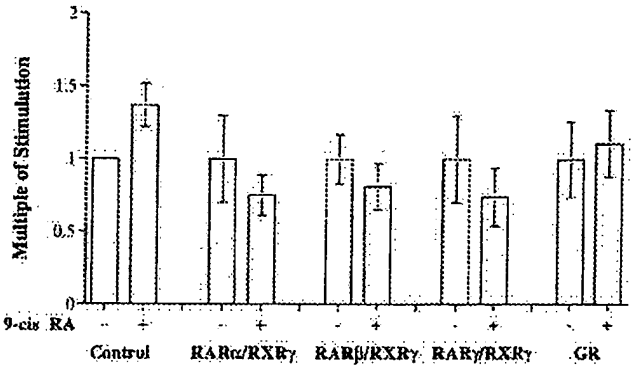



Fig. 7. Effect of RA and RARs/RXR-γ on thyroid transcription factor (TTF)-1 promoter. H441 cells were cotransfected with human TTF-1 1.7-kb luciferase reporter vector and RARs/RXR-γ or glucocorticoid-receptor expression plasmids. Transfected cells were treated with 10 μM 9-*cis*-RA or 50 nM dexamethasone. Luciferase activity was measured 48 h later. Activity of hTTF-1 1.7-kb luciferase reporter vector without 9-*cis*-RA treatment in absence of RARs/RXR-γ cotransfection was defined as 1. Activities were measured in light units of OD of β-galactosidase. Values are means ± SD; $n = 3$ experiments; each assay was performed in triplicate. ANOVA showed no significant stimulatory effect of 9-*cis*-RA, RARs/RAR-γ, and GR on human TTF-1 1.7-kb promoter, $P > 0.1$.

## DISCUSSION

In the present work, 9-*cis*-RA stimulated the transcriptional activity of hSP-B and mSP-B gene promoters in both human H441 and mouse MLE-15 cell lines. The observation that relatively high doses of RA were required for hSP-B promoter activation is consistent with a previous study (25) regarding the effects of RA on SP-B mRNA expression in cultured human fetal lung explants. 9-*cis*-RA also significantly enhanced endogenous SP-B mRNA expression and SP-B protein accumulation in H441 cells (Figs. 2 and 3). The present study also demonstrated that cotransfection of all three RAR isotypes in combination with RXR further enhanced RA treatment-dependent transactivation on the hSP-B promoter, supporting the concept that the effects of RA are mediated by RAR/RXR activation of the SP-B promoter. Detection of RAR (α and γ) and RXR (α) expression in H441 cells and the developing respiratory epithelium in vivo also supports their potential roles in the regulation of respiratory epithelial cell gene expression.

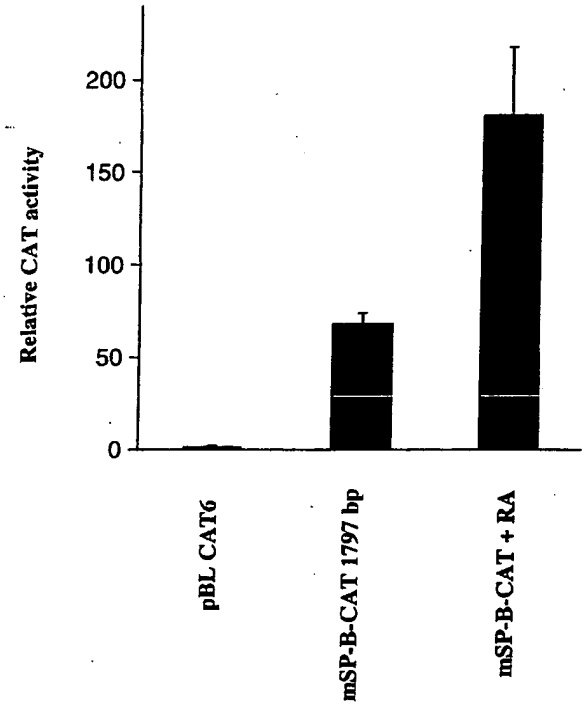



Fig. 6. RA stimulates murine (m) SP-B 1797 promoter activity. Murine MLE-15 cells were transfected with mSP-B-CAT 1797 reporter vector and followed by treatment with $10^{-5}$ M 9-*cis*-RA. Chloramphenicol transferase (CAT) activity was measured 48 h after treatment. Relative CAT activity is expressed compared with promotorless pBLCAT6 construct activity after normalization to β-galactosidase activity to correct for transfection efficiency. Values are means ± SD; $n = 3$ experiments, each performed in triplicate. ANOVA showed a significant stimulatory effect of 9-*cis*-RA on mSP-B 1797 promoter, $P < 0.05$.

The observation that RA enhanced SP-B mRNA and protein expression in H441 cells is consistent with previous findings from a study (25) of a human fetal lung explant culture. The RA-dependent induction in SP-B mRNA and protein is likely to be mediated, at least in part, by direct interaction of RAR with the SP-B gene promoter to enhance SP-B gene transcription. An RARE mediating the effects of RA on SP-B transcription was localized in the region −415 to −440 on the hSP-B promoter by EMSA. Deletion of this RARE resulted in loss of RA stimulation in the transient transfection studies, supporting the concept that RA and RAR/RXR heterodimers exert their effects directly on the hSP-B promoter. Although both TTF-1 and HNF-3β stimulate the hSP-B promoter, TTF-1 had a much stronger stimulatory effect (six- to sevenfold) on the hSP-B promoter than HNF-3β (around twofold stimulation) (42). The potential RAR sites were in close proximity to the distal TTF-1 clustered sites (40). We therefore tested whether RA enhances TTF-1 transcription, which would be more likely to enhance SP-B transcription than HNF-3β, which is much less active on the SP-B promoter. In contrast, cotransfection of RAR/RXR with the TTF-1 promoter construct did not alter its activity, although it remains possible that RA may alter TTF-1 expression or activity by other mechanisms. For example, a recent study (16) demonstrated

that the nuclear localization of TTF-1 and HNF-3β was affected by phorbol ester.

The distribution of RARs in developing fetal mouse lung was determined by in situ hybridization (9). Although RAR-β was expressed in the epithelium of proximal bronchi in *day 14.5* postconception embryos, RAR-α and -γ mRNAs were expressed homogeneously, even though weakly, in lung tissue (9). The critical roles of RAR-s in lung organogenesis were revealed by RAR gene double-knockout mice (23). In these mice, both the right and left lungs were either absent or markedly hypoplastic, indicating that RARs are required for normal lung formation. H441 cells share many features with bronchiolar respiratory epithelial cells from the distal conducting airway. The presence of RAR-α and -γ and RXR-α in H441 cells is consistant with their origin from distal respiratory epithelium and suggests their possible roles in bronchiolar cell differentiation and cell-type lineage maintenance.

Analysis of the GR-deficient mice revealed that a GR is required for lung maturation and perinatal survival (8). The GR-deficient mice die after birth of respiratory failure that is caused by a lack of inflation of the lung. However, SPs, including SP-B, are detected in the lung from the GR-deficient animals. In a previous study (28), dexamethasone stimulated SP-B mRNA and protein in H441 cells mediated by increasing SP-B mRNA stability. It has also been reported that dexamethasone stimulated SP-B transcription, although it remains unclear whether the effects of glucocorticoids are mediated directly by interaction of a GR with the SP-B promoter (1, 2, 10, 34). In the present study, dexamethasone repressed hSP-B-2240 transcription when cotransfected with a GR in H441 cells (Table 1). Thus the hSP-B-2240 region does not mediate the stimulatory effects of dexamethasone on SP-B expression in the H441 cell line.

RA plays a critical role both in lung organogenesis and in postnatal alveolarization (20, 21). All *trans*-RA caused a 50% increase in the number of alveoli in postnatal rats (20). Treatment of dexamethasone inhibited the formation of alveoli. Treatment with RA prevented the inhibitory effects of dexamethasone on alveolarization (20). RA also reversed elastase-induced pulmonary emphysema in adult rats, consistent with the potential roles of RA in the growth and differentiation of lung parenchyma.

In summary, the present study demonstrates a direct stimulatory effect of RA and RAR/RXR on SP-B gene transcription. RA and RAR/RXR increased SP-B gene transcription, mRNA accumulation, and SP-B synthesis in vitro.

We thank Dr. Susan Wert for assistance with photomicroscopy and Dr. Tim Weaver for providing proSP-B antibody. We thank Dr. Pierre Chambon for providing the retinoic acid-receptor and retinoid X-receptor plasmids and Dr. Ronald M. Evan for providing the glucocorticoid-receptor plasmid.

This work was supported by the American Lung Association (C. Yan) and National Heart, Lung, and Blood Institute Specialized Center of Research Grant HL-56387 (to J. A. Whitsett and C. Yan).

Address for reprint requests: C. Yan, Children's Hospital Medical Center, Division of Pulmonary Biology, TCHRF, 3333 Burnet Ave., Cincinnati, OH 45229-3039.

Received 15 October 1997; accepted in final form 14 April 1998.

## REFERENCES

1. **Ballard, P. L., R. Ertsey, L. W. Gonzales, and J. Gonzales.** Transcriptional regulation of human pulmonary surfactant proteins SP-B and SP-C by glucocorticoids. *Am. J. Respir. Cell Mol. Biol.* 14: 599–607, 1996.
2. **Beers, M. F., H. Shuman, H. G. Liley, J. Floros, L. W. Gonzales, N. Yue, and P. L. Ballard.** Surfactant protein B in human fetal lung: developmental and glucocorticoid regulation. *Pediatr. Res.* 38: 668–675, 1995.
3. **Bogue, C. W., H. C. Jacobs, D. W. Dynia, C. M. Wilson, and L. Gross.** Retinoic acid increases surfactant protein mRNA in fetal rat lung in culture. *Am. J. Physiol.* 271 (*Lung Cell. Mol. Physiol.* 15): L862–L868, 1996.
4. **Bohinski, R., R. Di Lauro, and J. A. Whitsett.** The lung-specific surfactant protein B gene promoter is a target for thyroid transcription factor 1 and hepatocyte nuclear factor 3, indicating common factors for organ-specific gene expression along the foregut axis. *Mol. Cell. Biol.* 14: 5671–5681, 1994.
5. **Bruno, M. A., R. J. Bohinski, J. E. Carter, K. A. Foss, and J. A. Whitsett.** Structure and function of the mouse surfactant protein B gene. *Am. J. Physiol.* 268 (*Lung Cell. Mol. Physiol.* 12): L381–L390, 1995.
6. **Cardoso, W. V., M. C. Williams, S. A. Mitsialis, M. Joyce-Brady, A. K. Rishi, and J. J. Brady.** Retinoic acid induces changes in the pattern of airway branching and alters epithelial cell differentiation in the developing lung in vivo. *Am. J. Respir. Cell Mol. Biol.* 12: 464–467, 1995.
7. **Clark, J. C., S. E. Wert, C. J. Bachurski, M. T. Stahlman, B. R. Stripp, T. E. Weaver, and J. A. Whitsett.** Targeted disruption of the surfactant protein B gene disrupts surfactant homeostasis, causing respiratory failure in newborn mice. *Proc. Natl. Acad. Sci. USA* 92: 7794–7798, 1995.
8. **Cole, T. J., J. A. Blendy, A. P. Monaghan, K. Krieglstein, W. Schmid, A. Aguzzi, G. Fantuzzi, E. Hummler, K. Unsicker, and G. Schutz.** Targeted disruption of the glucocorticoid receptor gene blocks adrenergic chromaffin cell development and severely retards lung maturation. *Genes Dev.* 9: 1608–1621, 1995.
9. **Dolle, P., E. Ruberte, P. Leroy, G. Morriss-Kay, and P. Chambon.** Retinoic acid receptors and cellular retinoid binding proteins I. A systematic study of their differential pattern of transcription during mouse organogenesis. *Development* 110: 1133–1151, 1990.
10. **Durham, P. L., C. L. Wohlford-Lenane, and J. M. Snyder.** Glucocorticoid regulation of surfactant-associated proteins in rabbit fetal lung in vivo. *Anat. Rec.* 237: 365–377, 1993.
11. **George, T. N., and J. M. Snyder.** Regulation of surfactant protein gene expression by retinoic acid metabolites. *Pediatr. Res.* 41: 692–701, 1997.
12. **Ghaffari, M., X. Zeng, J. A. Whitsett, and C. Yan.** Nuclear localization domain of thyroid transcription factor-1 in respiratory epithelial cells. *Biochem. J.* 328: 757–761, 1997.
13. **Ikeda, K., J. C. Clark, J. R. Shaw-White, M. T. Stahlman, C. J. Boutell, and J. A. Whitsett.** Gene structure and expression of human thyroid transcription factor-1 in respiratory epithelial cells. *J. Biol. Chem.* 270: 8108–8114, 1995.
14. **Kamei, Y., L. Xu, T. Heinzel, J. Torchia, R. Kurokawa, B. Gloss, S. C. Lin, R. A. Heyman, D. W. Rose, C. K. Glass, and M. G. Rosenfeld.** A CBP integrator complex mediates transcriptional activation and AP-1 inhibition by nuclear receptors. *Cell* 85: 403–414, 1996.
15. **Kastner, P., M. Mark, and P. Chambon.** Nonsteroid nuclear receptors: what are genetic studies telling us about their role in real life? *Cell* 83: 859–869, 1995.
16. **Kumar, A. S., V. C. Venkatesh, B. C. Planer, S. I. Feinstein, and P. L. Ballard.** Phorbol ester down-regulation of lung surfactant protein B gene expression by cytoplasmic trapping of thyroid transcription factor-1 and hepatocyte nuclear factor 3. *J. Biol. Chem.* 272: 20764–20773, 1997.
17. **Lazzaro, D., M. Price, M. D. Felice, and R. Di Lauro.** The transcription factor TTF-1 is expressed at the onset of thyroid and lung morphogenesis and in restricted regions of the foetal brain. *Development* 113: 1093–1104, 1991.

18. **Mangelsdorf, D. J., and R. M. Evans.** The RXR heterodimers and orphan receptors. *Cell* 83: 841–850, 1995.
19. **Mangelsdorf, D. J., C. Thummel, M. Beato, P. Herrlich, G. Schutz, K. Umesono, B. Blumberg, P. Kastner, M. Mark, P. Chambon, and R. M. Evans.** The nuclear receptor superfamily: the second decade. *Cell* 83: 835–839, 1995.
20. **Massaro, G. D., and D. Massaro.** Postnatal treatment with retinoic acid increases the number of pulmonary alveoli in rats. *Am. J. Physiol.* 270 (*Lung Cell. Mol. Physiol.* 14): L305–L310, 1996.
21. **Massaro, G. D., and D. Massaro.** Retinoic acid treatment abrogates elastase-induced pulmonary emphysema in rats. *Nat. Med.* 3: 675–677, 1997.
22. **Masuyama, H., Y. Hiramatsu, and T. Kudo.** Effect of retinoids on fetal lung development in the rat. *Biol. Neonate* 67: 264–273, 1995.
23. **Mendelsohn, C., D. Lohnes, D. Decimo, T. Lufkin, M. LeMeur, P. Chambon, and M. Mark.** Function of the retinoic acid receptors (RARs) during development (II). Multiple abnormalities at various stages of organogenesis in RAR double mutants. *Development* 120: 2749–2771, 1994.
24. **Mengus, G., M. May, L. Carre, P. Chambon, and I. Davidson.** Human $TAF_{II}135$ potentiates transcriptional activation by the AF-2s of the retinoic acid, vitamin D3, and thyroid hormone receptors in mammalian cells. *Genes Dev.* 11: 1381–1395, 1997.
25. **Metzler, M., and J. M. Snyder.** Retinoic acid differentially regulates expression of surfactant-associated proteins in human fetal lung. *Endocrinology* 133: 1990–1998, 1993.
26. **Nogee, L. M., D. E. deMello, L. P. Dehner, and H. R. Colten.** Brief report: deficiency of pulmonary surfactant protein B in congenital alveolar proteinosis. *N. Engl. J. Med.* 328: 406–410, 1993.
27. **Nogee, L. M., G. Garnier, H. C. Dietz, L. Singer, A. M. Murphy, D. E. deMello, and H. R. Colter.** A mutation in the surfactant protein B gene responsible for fatal neonatal respiratory disease in multiple kindreds. *J. Clin. Invest.* 93: 1860–1863, 1994.
28. **O'Reilly, M. A., J. C. Clark, and J. A. Whitsett.** Glucocorticoids enhance pulmonary surfactant protein B gene transcription. *Am. J. Physiol.* 260 (*Lung Cell. Mol. Physiol.* 4): L37–L43, 1991.
29. **Pryhuber, G. S., S. L. Church, T. Kroft, A. Panchal, and J. A. Whitsett.** 3′-Untranslated region of SP-B mRNA mediates inhibitory effects of TPA and TNF-α on SP-B expression. *Am. J. Physiol.* 267 (*Lung Cell. Mol. Physiol.* 11): L16–L24, 1994.
30. **Pryhuber, G. S., M. A. O'Reilly, J. C. Clark, W. M. Hull, I. Fink, and J. A. Whitsett.** Phorbol ester inhibits surfactant protein SP-A and SP-B expression. *J. Biol. Chem.* 265: 20822–20828, 1990.
31. **Rochette-Egly, C., S. Adam, M. Rossignol, J. M. Egly, and P. Chambon.** Stimulation of RARα activation function AF-1 through binding to the general transcription factor TFIIH and phosphorylation by CDK7. *Cell* 90: 97–107, 1997.
32. **Salbert, G., A. Fanjul, F. J. Piedrafita, X. P. Lu, S. J. Kim, P. Tran, and M. Pfahl.** Retinoic acid receptors and retinoid X receptor α regulate the transforming growth factor-β1 promoter by antagonizing AP-1 activity. *Mol. Endocrinol.* 10: 1347–1356, 1993.
33. **Shenai, J. P., F. Chytil, and M. T. Stahlman.** Vitamin A status of neonates with bronchopulmonary dysplasia. *Pediatr. Rev.* 19: 185–188, 1985.
34. **Venkatesh, V. C., D. M. Iannuzzi, R. Ertsey, and P. L. Ballard.** Differential glucocorticoid regulation of the pulmonary hydrophobic surfactant proteins SP-B and SP-C. *Am. J. Respir. Cell Mol. Biol.* 8: 222–228, 1993.
35. **Venkatesh, V. C., B. C. Planer, M. Schwartz, J. N. Vanderbilt, R. T. White, and P. L. Ballard.** Characterization of the promoter of human pulmonary surfactant protein B gene. *Am. J. Physiol.* 268 (*Lung Cell. Mol. Physiol.* 12): L674–L682, 1995.
36. **Whitsett, J. A., L. M. Nogee, T. E. Weaver, and A. D. Horowitz.** Human surfactant protein B: structure, function, regulation, and genetic disease. *Physiol. Rev.* 75: 749–757, 1995.
37. **Wikenheiser, K. A., D. K. Vorbroker, W. R. Rice, J. C. Clark, C. J. Bachurski, H. K. Oie, and J. A. Whitsett.** Production of immortalized distal respiratory epithelial cell lines from surfactant protein C/simian virus 40 large tumor antigen transgenic mice. *Proc. Natl. Acad. Sci. USA* 90: 11029–11033, 1993.
38. **Wispe, J. R., J. C. Clark, B. B. Warner, D. Fajardo, W. E. Hull, R. B. Holtzman, and J. A. Whitsett.** Tumor necrosis factor-alpha inhibits expression of pulmonary surfactant protein. *J. Clin. Invest.* 86: 1954–1960, 1990.
39. **Yan, C., P. B. Sehgal, and I. Tamm.** Signal transduction pathways in the induction of 2′,5′-oligoadenylate synthetase gene expression by interferon α/β. *Proc. Natl. Acad. Sci. USA* 86: 2243–2247, 1989.
40. **Yan, C., Z. Sever, and J. A. Whitsett.** Upstream enhancer activity in the human surfactant protein B gene is mediated by thyroid transcription factor 1. *J. Biol. Chem.* 270: 24852–24857, 1995.
41. **Yan, C., and I. Tamm.** Molecular cloning and characterization of additional factors that bind to the interferon-α/β response element B of the murine (2′5′)oligoadenylate synthetase ME-12 gene. *Proc. Natl. Acad. Sci. USA* 89: 8859–8863, 1992.
42. **Yan, C., and J. A. Whitsett.** Protein kinase A activation of the surfactant protein B gene is mediated by phosphorylation of thyroid transcription factor 1. *J. Biol. Chem.* 272: 17327–17332, 1997.

09/919195

| | |
|---|---|
| ACCESSION NUMBER: | 131:69157 CA |
| TITLE: | Inhibition of hSP-B promoter in respiratory epithelial cells by a dominant negative retinoic acid receptor |
| AUTHOR(S): | Ghaffari, Manely; Whitsett, Jeffrey A.; Yan, Cong |
| CORPORATE SOURCE: | Division of Pulmonary Biology, Children's Hospital Medical Center, Cincinnati, OH, 45229-3039, USA |
| SOURCE: | American Journal of Physiology (**1999**), **276**(3, Pt. 1), **L398**-L404<br>CODEN: AJPHAP; ISSN: 0002-9513 |
| PUBLISHER: | American Physiological Society |
| DOCUMENT TYPE: | Journal |
| LANGUAGE: | English |
| REFERENCE COUNT: | 48 THERE ARE 48 CITED REFERENCES AVAILABLE FOR THIS RECORD. ALL CITATIONS AVAILABLE IN THE RE FORMAT |

# Inhibition of hSP-B promoter in respiratory epithelial cells by a dominant negative retinoic acid receptor

MANELY GHAFFARI, JEFFREY A. WHITSETT, AND CONG YAN
*Division of Pulmonary Biology, Children's Hospital Medical Center, Cincinnati, Ohio 45229-3039*

**Ghaffari, Manely, Jeffrey A. Whitsett, and Cong Yan.** Inhibition of hSP-B promoter in respiratory epithelial cells by a dominant negative retinoic acid receptor. *Am. J. Physiol.* 276 (*Lung Cell. Mol. Physiol.* 20): L398–L404, 1999.— Retinoic acid (RA) receptors (RARs) belong to the nuclear hormone receptor superfamily and play important roles in lung differentiation, growth, and gene regulation. Surfactant protein (SP) B is a small hydrophobic protein synthesized and secreted by respiratory epithelial cells in the lung. Expression of the *SP-B* gene is modulated at the transcriptional and posttranscriptional levels. In the present work, immunohistochemical staining revealed that RAR-α is present on *day 14.5* of gestation in the fetal mouse lung. To assess whether RAR is required for *SP-B* gene transcription, a dominant negative mutant human (h) RAR-α403 was generated. The hRAR-α403 mutant was transcribed and translated into the truncated protein product by reticulocyte lysate in vitro. The mutant retained DNA binding activity in the presence of retinoid X receptor-γ to an RA response element in the hSP-B promoter. When transiently transfected into pulmonary adenocarcinoma epithelial cells (H441 cells), the mutant hRAR-α403 was readily detected in the cell nucleus. Cotransfection of the mutant hRAR-α403 repressed activity of the hSP-B promoter and inhibited RA-induced surfactant proprotein B production in H441 cells, supporting the concept that RAR is required for *hSP-B* gene transcription in vitro.

human surfactant protein B; lung development; nuclear receptors

SURFACTANT PROTEIN (SP) B is a 79-amino acid amphipathic peptide produced by the proteolytic cleavage of surfactant proprotein (proSP) B in type II epithelial cells in the alveoli of the lung. The SP-B peptide is stored in lamellar bodies and secreted with phospholipids into the airway lumen. The function of SP-B is to stabilize the surfactant membrane layer and facilitate the spreading of phospholipids, preventing collapse during the respiratory cycle. SP-B is an essential component of surfactant and is required for postnatal respiratory adaptation (43). Mutations in the *SP-B* gene in both humans and mice cause respiratory failure after birth (8, 29, 30).

It has been well established that retinoic acid (RA) receptors (RARs) play critical roles in proliferation, differentiation, and apoptosis in a variety of epithelial cells. Recently, RAR-α and -γ and retinoid X receptor (RXR)-α were detected in the H441 cell line, which is derived from pulmonary adenocarcinoma epithelial cells. In H441 cells, RA and RARs stimulated SP-B promoter activity, mRNA accumulation, and protein production (12, 13, 45). Increases in SP-B mRNA and protein accumulation in fetal lung explants have also been observed (2, 28). RA stimulation of the human (h) *SP-B* gene is mediated through direct binding of the RAR/RXR heterodimer to the SP-B promoter (45). The region between −375 to −500 bp of the hSP-B promoter was identified as the responsible sequence for RAR binding and transactivation (45).

Lung development is dependent on expression of RARs (10, 26). RAR-α, -β, and -γ belong to the superfamily of nuclear receptors. They form heterodimers with the RXR, bind to the RA response element (RARE) on promoter regions of target genes, and exert stimulatory effects after binding to their ligand RAs (19, 21, 22). Expression of all three isotypes of RAR was previously detected in the developing lung by in situ hybridization or RT-PCR in the mouse and rat (10, 25). RAR-α and -β null mutant mice died in utero and had severely hypoplastic lungs (26). Lung organogenesis is dependent on interactions between mesenchymal and epithelial cells. Several studies (3, 6, 28) in lung buds indicated that lung branching morphogenesis and differentiation were strongly influenced by RA in vitro. Recent studies (23, 24) demonstrated that RA also influences alveolarization. Treatment of rats with all *trans*-RA increased the number of alveoli and reversed an alveolar disorder caused by elastase-induced pulmonary emphysema in animals (23, 24).

RARs consist of a DNA binding domain that contains $Zn^{2+}$ finger motifs, ligand binding and dimerization domains, a ligand-independent transcriptional activation (AF-1) domain, a ligand-dependent transcriptional activation (AF-2) domain, and an F region (19, 21, 22). Through these various domains, RARs interact with other transcriptional and signaling cofactors, including p160/SRC-I/TIF-II/Rac-III, CBP/p300, AP-1, TFIIH, and $TAF_{II}135$ (7, 15, 16, 18, 27, 31, 35, 39, 42). Through structure-function studies, dominant negative mutants of RARs have been developed and characterized (9, 11). Removal of the COOH-terminal AF-2 domain of RARs yields dominant negative mutant receptors in cultured animal cells. The integrity of both the DNA binding and heterodimerization functions of RARs is required for the dominant negative effect. The dominant negative mutant of RAR-α blocks wild-type RAR function and has been successfully used in transgenic mice (17).

To further elucidate the biological functions and mechanism of RAR-α in the regulation of *hSP-B* gene expression, a dominant negative mutant, hRAR-α403, was generated and expressed in H441 cells with a mammalian cell expression vector. This was based on an observation that RAR-α was detected on *day 14.5* of gestation in the fetal mouse lung. The mutant RAR-α

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

protein was expressed in H441 cells and localized to the nucleus. The mutant hRAR-α403 protein retained DNA binding activity on the RARE of the hSP-B promoter. Cotransfection of the mutant hRAR-α403 strongly inhibited transcription of the hSP-B promoter by luciferase reporter assay and proSP-B production in H441 cells.

## METHODS AND MATERIALS

*Cell culture.* Human pulmonary adenocarcinoma (H441) cells were cultured in RPMI 1640 medium (GIBCO BRL, Grand Island, NY) supplemented with 10% fetal calf serum, glutamine, and penicillin-streptomycin. Cells were maintained at 37°C in 5% $CO_2$-air and passaged weekly.

*Plasmid constructs.* The hSP-B 500-bp promoter (hSP-B-500) was made as previously described (46). hRAR-α403-FLAG was generated by PCR with synthetic oligonucleotide primers, with an hRAR-α/pSG5 construct as a template (kindly provided by Dr. Pierre Chambon, Institut de Génétique et de Biologie Moléculaire et Cellulaire, Strassbourg, France). The upstream primer with the *Eco*R I site and the Kozak sequence was 5′-GCGGAATTCGCCACCATGGCCAGCAACAGCAGCTCC-3′. The downstream primer with the *Xba* I site and the FLAG sequence (underlined) was 5′-CTCGCTCTAGATTATCA-CTTGTCATCGTCGTCCTTGTAGTCCGGGATCTCCATCTT-CAGCGT-3′. The PCR products were digested with *Eco*R I and *Xba* I restriction enzymes and ligated with *Eco*R I-*Xba* I-digested PCR3.0 luciferase reporter plasmid that contains the cytomegalovirus (CMV) promoter (Invitrogen, Carlsbad, CA). The correctness of hRAR-α403-FLAG/PCR3.0 was confirmed by DNA sequencing. hRAR-α462-FLAG/PCR was generated the same way except that the downstream primer was 5′-CTCGCTCTAGATTATCACTTGTCATCGTCGTCCTTGTA-GTCCGGGGAGTGGGTGGCCGGGCT-3′. All primers were made by GIBCO BRL.

*In vitro transcription, translation, and Western blot analysis.* hRAR-α403-FLAG/PCR3.0 and hRAR-α462-FLAG/PCR3.0 were transcribed and translated with the TNT T7 Quick Coupled Transcription/Translation System (Promega, Madison, WI) at 30°C for 90 min. As a positive control, thyroid transcription factor (TTF)-1-FLAG/PCR3.0 was also transcribed and translated. The negative control was the PCR3.0 empty vector. The programmed products were separated on a 10% polyacrylamide gel and subsequently transferred to a nitrocellulose membrane. Western blot analysis with an anti-FLAG monoclonal antibody was performed as described previously (14).

*Electrophoretic mobility shift assay.* A previously described RARE oligo probe of the hSP-B promoter was used for an electrophoretic mobility shift assay (EMSA) study (45). In vitro transcribed and translated hRAR-α403 and hRXR-γ proteins were incubated with the radiolabeled probe and separated by 4% nondenaturing gel as described previously (46). Antibody recognizing RAR (5 μg; Santa Cruz Biotechnology, Santa Cruz, CA) was used for identifying the hRAR-α403/hRXR-γ complex in an EMSA study.

*Transient transfection and luciferase assays.* Cotransfection of hRAR-α403-FLAG with hSP-B-500 and a luciferase assay were performed in H441 cells as previously described (45, 47). The pCMV-β-Gal plasmid was cotransfected for normalizing transfection efficiency. Each experiment was repeated at least three times. Significance of the inhibitory effects of hRAR-α403-FLAG was determined by one-way ANOVA with the SigmaStat program.

*Immunohistochemistry and immunofluorescent staining.* Immunohistochemical staining of H441 cells transfected with hRAR-α403-FLAG/PCR3.0, hRAR-α462-FLAG/PCR3.0, and TTF-1-FLAG/PCR3.0 with the anti-FLAG antibody (Kodak, New Haven, CT) was performed as previously described (45). In the control cells, no specific antibody was added. Immunofluorescent staining of H441 cells transfected without and with hRAR-α403-FLAG/PCR3.0 was performed as previously described (14). Expression of hRAR-α403-FLAG/PCR3.0 was detected with the anti-FLAG antibody and Texas Red-conjugated goat anti-rabbit secondary IgG (Jackson ImmunoResearch Laboratories, West Grove, PA). Expression of proSP-B was detected with a proSP-B polyclonal antibody and FITC-conjugated goat anti-mouse IgG secondary IgG (Jackson ImmunoResearch Laboratories). All *trans*-RA was purchased from Sigma (St. Louis, MO).

## RESULTS

*Expression of RAR-α in developing lung.* We previously detected RAR-α expression in respiratory epithelial H441 cells. To assess which isoforms of RAR are expressed in the developing lung, tissue sections from a fetal mouse lung on *day 14.5* of gestation were immunohistochemically stained with an antibody recognizing RAR-α. Figure 1 shows staining of RAR-α in the epithelium of the developing mouse fetal lung by the anti-RAR-α polyclonal antibody. Based on this result, the α isotype of RAR was chosen to make a dominant negative mutant for further study of the hSP-B promoter.

*Construction of dominant negative hRAR-α403-FLAG.* To test whether expression of a dominant negative mutant of RAR-α inhibited the hSP-B promoter, the mutant hRAR-α403-FLAG and the wild-type hRARa462-FLAG were inserted into the mammalian expression vector PCR3.0 as illustrated in Fig. 2. The mutant contains the AF-1, DNA binding domain, and ligand binding and dimerization domains of hRAR-α but lacks the AF-2 and F domains. A Kozak sequence (ACCATGTCG) was included at the $NH_2$ terminus of hRAR-α403 and hRAR-α462 to enhance the efficiency of translation. A FLAG sequence was included at the COOH terminus of hRAR-α403 and hRAR-α462 and

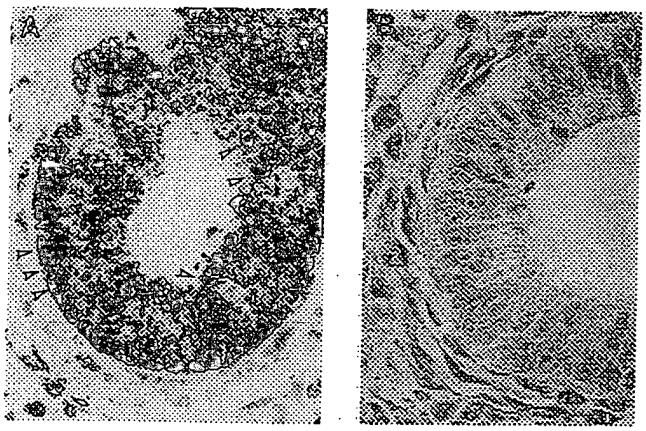



Fig. 1. Retinoic acid (RA) receptor (RAR)-α protein expression in *day 14.5* developing lung. Tissue sections from a fetal mouse lung on *day 14.5* of gestation were immunohistochemically stained with an antibody recognizing RAR-α (*A*). Arrows, stained cells in developing fetal lung epithelium. Control tissue section was stained without RAR-α antibody (*B*).

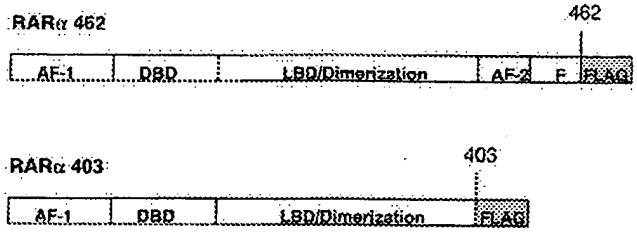



Fig. 2. Construction of human (h) RAR-α403-FLAG/PCR3.0 and hRAR-α403-FLAG/PCR3.0. Full-length hRAR-α462 and dominant negative hRAR-α403 lacking ligand-dependent transcriptional activation domain (AF-2) were isolated by PCR and inserted into mammalian cell expression vector PCR3.0. A Kozak sequence was included at the $NH_2$ terminus, and a FLAG sequence was included at the COOH terminus of hRAR-α403 and hRAR-α462. AF-1, ligand-independent transcriptional activation domain; DBD, DNA binding domain; LBD/dimerization, ligand binding and dimerization domains; F, F domain. Nos. above bars, no. of bp.

was used to distinguish the mutant receptor from endogenous RAR-α.

*Expression of hRAR-α403-FLAG and hRAR-α462-FLAG in vitro.* To test whether the hRAR-α403-FLAG/PCR3.0 and the wild-type hRAR-α462-FLAG/PCR3.0 vectors generated protein products of correct sizes, the constructs were transcribed and translated in vitro in the rabbit reticulocyte lysate system. The products of the programmed lysates were separated on a 10% polyacrylamide gel and analyzed by Western blot with the anti-FLAG antibody. Both the full-length hRAR-α462-FLAG and the short-form hRAR-α403-FLAG were produced in the reticulocyte lysate system (Fig. 3). Because TTF-1-FLAG/PCR3.0 was well characterized previously (14), it was also transcribed and translated in vitro as a positive control. In the negative control, the reticulocyte lysate revealed no FLAG fusion protein.

*Expression of hRAR-α403-FLAG in H441 cells.* Expression of the hRAR-α403-FLAG and hRAR-α462-FLAG vectors in H441 cells was assessed. The constructs hRAR-α403-FLAG/PCR3.0, hRAR-α462-FLAG/PCR3.0, and TTF-1-FLAG/PCR3.0 (positive control) were transiently transfected into H441 cells. Immunohistochemical analysis with an anti-FLAG antibody revealed strong nuclear staining of the FLAG epitope for all three constructs (Fig. 4). Deletion of the AF-2 and F domains did not change hRAR-α403 expression and nuclear localization in H441 cells. Untransfected H441 cells did not stain with the anti-FLAG antibody.

*DNA binding activity of hRAR-α403-FLAG on RARE of the hSP-B promoter.* Next, in vitro transcribed and translated hRAR-α462-FLAG and hRAR-α403-FLAG fusion proteins were assessed for their DNA binding activities. As shown in Fig. 5, hRAR-α462-FLAG, hRAR-α403-FLAG, or hRXR-γ-FLAG fusion protein alone did not form DNA-protein complexes with RARE of the hSP-B promoter. This is due to their low DNA binding affinity. Yan et al. (45) previously reported that when higher concentrations of purified bacteria-expressed hRAR-γ-glutathione *S*-transferase (GST) fusion protein were used, interaction between hRAR-γ-GST and RARE was observed. In contrast, formation of hRAR-α462-FLAG/hRXR-γ-FLAG and hRAR-α403-FLAG/hRXR-γ-FLAG heterodimers with high DNA binding affinity generated detectable DNA-protein complexes with RARE (Fig. 5). Binding of hRAR-α462-FLAG/hRXR-γ-FLAG and hRAR-α403-FLAG/hRXR-γ-FLAG complexes with RARE was blocked by the anti-RAR antibody. This is probably due to interference of the RAR antibody with either DNA binding or dimerization of the hRAR-α403-FLAG/hRXR-γ or hRAR-α462-FLAG/hRXR-γ heterodimer. Thus, like the wild-type hRAR-α in the presence of hRXR, the mutant hRAR-α403 retained DNA binding activity on RARE of the hSP-B promoter. Nonspecific bands observed in EMSA, which did not contain the hRAR-α462-FLAG and hRAR-α403-FLAG fusion proteins, were also detected in the control samples (PCR3.0 empty vector). Nonspecific bands were not changed by the RAR antibody.

*Inhibitory effect of hRAR-α403-FLAG on the hSP-B promoter in H441 cells.* Cotransfection of hRAR-α403-FLAG/PCR3.0 with the hSP-B-500 luciferase reporter gene into H441 cells demonstrated that the mutant hRAR-α strongly inhibited luciferase reporter activity (Fig. 6). In contrast, the wild-type hRAR-α stimulated the hSP-B luciferase reporter gene (45). The inhibitory effects of the mutant hRAR-α403 on all *trans*-RA-treated and untreated hSP-B-500 were dose dependent. Interestingly, the mutant hRAR-α did not completely

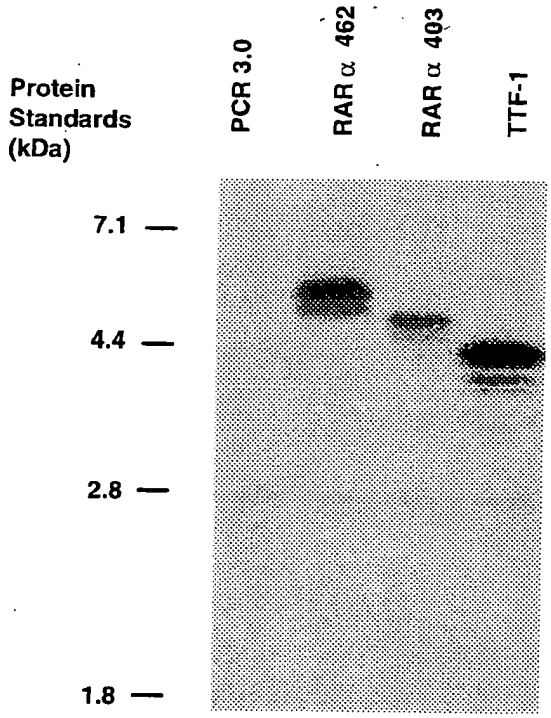



Fig. 3. In vitro transcription and translation of hRAR-α462-FLAG and hRAR-α403-FLAG. Mammalian expression vectors hRAR-α462-FLAG/PCR3.0 and hRAR-α403-FLAG/PCR3.0 were transcribed and translated in vitro by rabbit reticulocyte lysate system. Synthesized products were electrophoresed and analyzed by Western blot with anti-FLAG antibody. Thyroid transcription factor (TTF)-1-FLAG/PCR3.0 was used as a positive control. Size of each synthesized protein was matched well to anticipated molecular mass (nos. at *left* × 10). Empty PCR3.0 was used as a negative control.

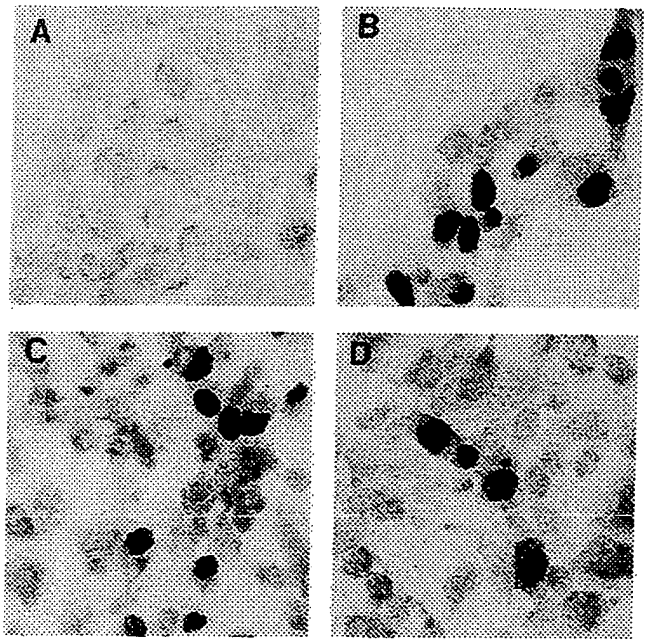



Fig. 4. Immunohistochemical staining of hRAR-α462 and hRAR-α403 in H441 cells. H441 cells were transfected with empty plasmid PCR3.0 (*A*), TTF-1-FLAG/PCR3.0 (*B*), hRAR-α462-FLAG/PCR3.0 (*C*), and hRAR-α403-FLAG/PCR3.0 (*D*) for 48 h. Transfected cells were immunostained with anti-FLAG monoclonal antibody. Cells transfected with hRAR-α and TTF-1 FLAG fusion protein constructs were immunostained in the nucleus.

abolish the hSP-B-500 activity. The residual activity of the hSP-B-500 seen in the presence of the mutant hRAR-α was similar to that of the basal activity of the hSP-B 218-bp promoter luciferase reporter gene, suggesting that RAR is not required for basal transcription activity of the hSP-B promoter. Therefore, RAR stimulates the hSP-B promoter through RARE located in the upstream enhancer region as previously identified (46). All *trans*-RA significantly reversed some of the inhibitory effect of hRAR-α403. This was probably due to the activation of endogenous RAR. Treatment with 9-*cis*-RA had the same reversal effect as all *trans*-RA (data not shown).

*Inhibitory effect of hRAR-α403-FLAG on RA-induced proSP-B production in H441 cells.* The effect of hRAR-α403-FLAG on proSP-B was also examined in H441 cells with double immunofluorescence staining analysis. All *trans*-RA strongly stimulated proSP-B synthesis in H441 cells as detected by a proSP-B polyclonal antibody (Fig. 7*A*), in agreement with previous observations that 9-*cis*-RA stimulated proSP-B staining in H441 cells (45). This is also in agreement with the observation that both all *trans*-RA and 9-*cis*-RA significantly increased SP-B protein levels in human fetal lung explants (13). When cells were transfected with hRAR-α403-FLAG as detected by a FLAG monoclonal antibody, no stimulation of proSP-B by all *trans*-RA was observed in transfected cells (Fig. 7, *B* and *C*).

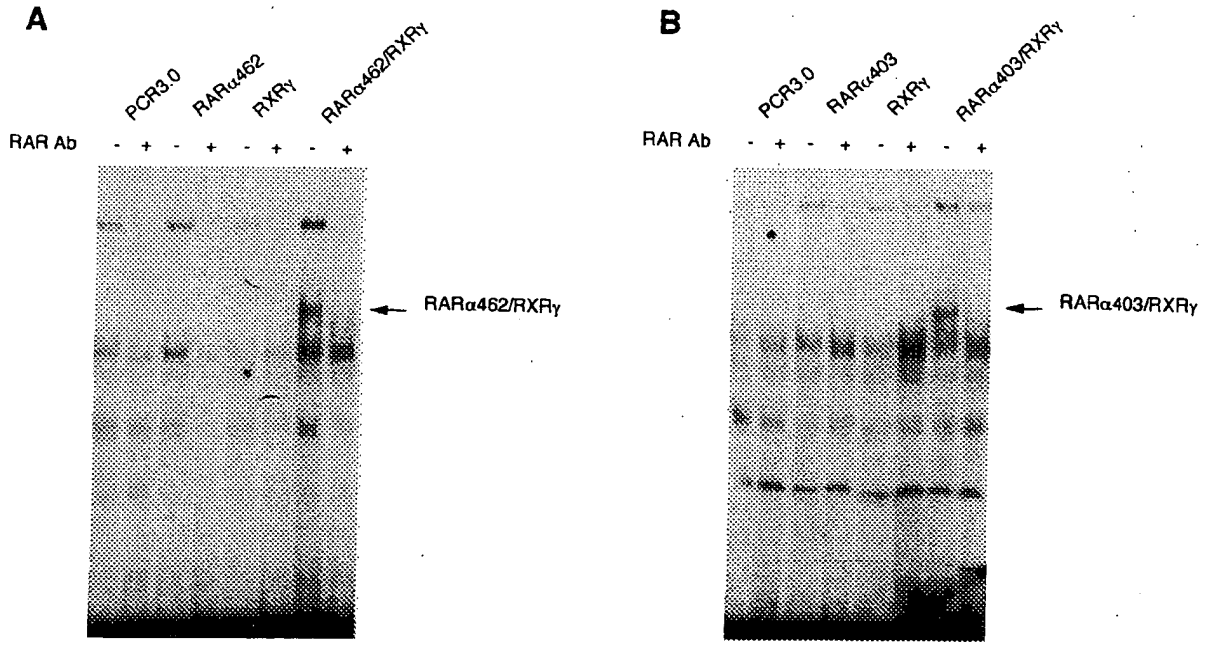



Fig. 5. Electrophoretic mobility shift assay (EMSA) study of hRAR-α403-FLAG with RA response element (RARE) of human surfactant protein (hSP)-B promoter. In vitro transcribed and translated hRAR-α462-FLAG (*A*), hRAR-α403-FLAG (*B*), and human retinoid X receptor (RXR)-γ-FLAG fusion proteins were incubated with radiolabeled hSP-B RARE in absence (−) and presence (+) of RAR antibody (Ab). Controls include in vitro transcribed and translated proteins from parent PRC3.0 empty vector. There were several nonspecific bands present in all lanes including control (PCR3.0). Specific bands were detected in hRAR-α462-FLAG/hRXR-γ-FLAG and hRAR-α403-FLAG/hRXRγ-FLAG lanes, which were blocked by RAR Ab.

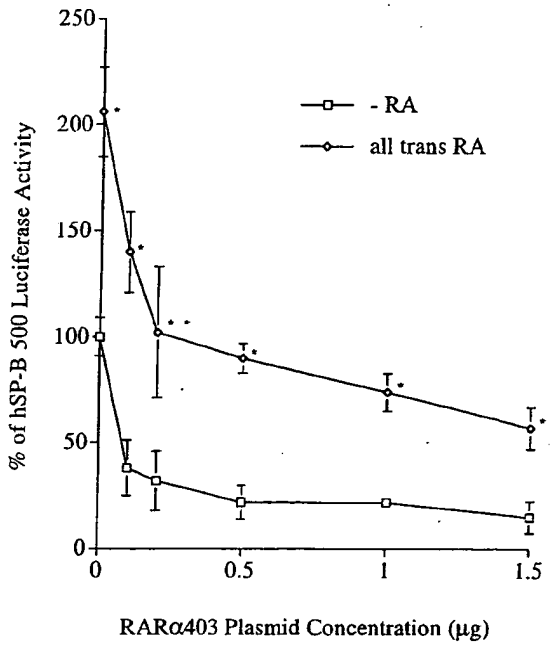



Fig. 6. hRAR-α403-FLAG inhibitory effect on hSP-B 500-bp promoter fragment (hSP-B-500). H441 cells were cotransfected with various concentrations of hRAR-α403-FLAG/PCR3.0 and 0.2 μg of hSP-B-500 luciferase reporter vector. Luciferase activity was measured 48 h after treatment. hSP-B-500 activity without hRAR-α403-FLAG/PCR3.0 cotransfection was defined as 1. Activities were measured in light units/optical density of β-galactosidase. Values are means ± SD; $n = 3$ experiments. ANOVA analysis showed significant inhibitory effect ($P < 0.05$). Significant difference from untreated samples: $^{*}r < 0.01$; $^{**}r < 0.025$ (both by ANOVA).

## DISCUSSION

SP-B mRNA is expressed selectively in bronchiolar and alveolar cells, and its expression is influenced at both the transcriptional and posttranscriptional levels (1, 2, 4, 20, 32–34, 40, 41, 44, 46, 48). RA stimulates production of SP-B mRNA and increases transcriptional activity (3, 12, 13, 28, 45). Stimulatory effects of RA are mediated by direct DNA binding of liganded RAR/RXR to an RARE on the hSP-B promoter (45). One approach to elucidate the biochemical and physiological functions of RAR in the regulation of *SP-B* gene transcription and homeostasis is to utilize dominant negative RAR derivatives. To further support the notion that RAR is required for full activation of the SP-B promoter, a dominant negative hRAR-α403-FLAG mutant was generated by deleting the RAR-α AF-2 domain (Fig. 2). The RAR-α dominant negative mutant was selected in the present study because immunohistochemical staining indicated expression of RAR-α in the fetal lung in vivo (Fig. 1) and in respiratory epithelial H441 cells in vitro (45). It is worthwhile to notice that only a certain population of progenitor epithelial cells was stained with the RAR-α antibody. This may imply that this group of progenitor cells will have a different fate during lung differentiation from cells without RAR-α expression.

The AF-2 domain of RAR is a ligand-dependent transactivation domain located in the COOH-terminal part of RAR. The AF-2 domain is highly conserved in many members of the nuclear receptor family and is indispensable for the ligand-mediated function. This conserved domain can be swapped between nuclear receptors without affecting the ligand dependency for transactivation (19, 21, 22). The COOH terminus of the domain (AF-2-AD) forms an amphipathic α-helical structure (5). It is proposed that hydrophobic residues of the AF-2-AD helix participate in ligand binding and that the charged residues of the AF-2-AD helix mediate protein-protein interactions with cofactors (see Ref. 16 for a review). Point mutagenesis of the hydrophobic or charged residues of this domain reduced its transactivation activity (11). After binding to RARE on the target genes, RARs interact with many coactivators (7, 15, 16, 18, 27, 31, 35, 39, 42). RARs and coactivators synergistically interact with each other to stimulate gene transcription. The AF-2 domain of RAR plays essential roles in mediating protein-protein interaction with coactivators. The present studies demonstrate that deletion of the AF-2 domain abrogated the hRAR-α stimulatory effect on the hSP-B promoter, suggesting that the AF-2 domain plays a critical role in activation of the hSP-B promoter in respiratory epithelial cells.

RAR-α, -β, and -γ can be converted into potent dominant negative transcriptional regulators that block the wild-type RAR function by deletion of the AF-2 domain and actively repress the basal transcription level of target promoters (9). Although deletion of AF-2 domain abrogated RAR transactivation activity of the hSP-B promoter (Fig. 6), it did not alter DNA binding of

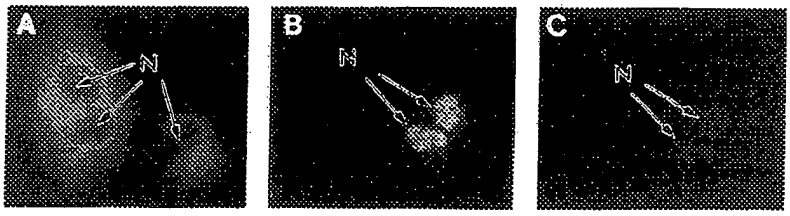



Fig. 7. hRAR-α403-FLAG inhibitory effects of all *trans*-RA on surfactant proprotein (proSP) B. *A*: H441 cells were treated with $10^{-5}$ M all *trans*-RA. Cells were stained with proSP-B polyclonal antibody and Texas Red-conjugated goat anti-rabbit secondary IgG. N, nuclei. *B*: H441 cells were transfected with hRAR-α403-FLAG and treated with $10^{-5}$ M all *trans*-RA. Expression of hRAR-α403-FLAG in the nuclei was detected with FLAG monoclonal antibody and FITC-conjugated goat anti-mouse IgG secondary IgG. *C*: cells in *B* were stained with proSP-B polyclonal antibody and Texas Red-conjugated goat anti-rabbit secondary IgG. No RA-induced proSP-B production was detected in cells expressing hRAR-α403-FLAG protein.

RAR to RARE of the hSP-B promoter (Fig. 5). hRAR-α403 forms an inactive hRAR-α403/hRXR heterodimer that blocks formation of an active hRAR-α/hRXR heterodimer to directly inhibit *hSP-B* gene expression. The inhibitory effect of hRAR-α403 on the hSP-B promoter might also result from indirect effects. This is evidenced by the observation that all *trans*-RA significantly reversed the inhibitory effect of hRAR-α403 on the hSP-B promoter. The mutant RAR-α protein was readily detected after transient transfection into H441 cells by immunohistochemical staining (Fig. 4), suggesting its stability and appropriate translocation to the nucleus.

The finding that the *SP-B* gene is a downstream target of RAR in pulmonary respiratory epithelial cells is important for understanding the metabolism and homeostasis of SP-B in prenatal and postnatal development of the lung. The function of the newborn lung is dependent on the differentiation of respiratory epithelial cells and the synthesis and secretion of surfactant lipids and proteins into the air space. Pulmonary surfactant is composed of lipids and proteins that reduce surface tension at the air-liquid interface in the alveoli. SPs, including SP-A, SP-B, SP-C, and SP-D, are synthesized primarily by type II or bronchiolar epithelial cells and play critical roles in maintaining stability of the surfactant layer. Lack of pulmonary surfactant leads to alveolar collapse and epithelial cell lysis in respiratory distress syndrome, a major cause of morbidity and mortality in preterm infants. Bronchopulmonary dysplasia (BPD) is a chronic lung disease that often occurs in preterm infants as a result of prolonged and high inspired oxygen concentrations, barotrauma from mechanical ventilation, hyaline membrane disease, and secondary infection with prolonged tracheal intubation. Studies (36–38) have shown that vitamin A supplementation from the early postnatal period could reduce the morbidity associated with BPD in preterm infants. Studies (45; present study) showed that the vitamin A derivatives RA and RAR stimulate *SP-B* gene and protein expression in pulmonary epithelial respiratory cells, supporting the recent findings (23, 24) that RA plays a critical role in postnatal alveolarization in vivo.

We thank Angela Naltner for critical reading of the manuscript. We thank Xin Zeng for providing fetal lung tissue sections and Dr. Jacquelyn A. Huffman Reed for helping with statistical analysis. We thank Dr. Pierre Chambon for providing the human retinoic acid receptor-α and human retinoid X receptor-γ plasmids.

This work was supported by the American Lung Association (C. Yan) and National Heart, Lung, and Blood Institute Specialized Center of Research Grant HL-56387 (to J. A. Whitsett and C. Yan).

Address for reprint requests: C. Yan, Children's Hospital Medical Center, Division of Pulmonary Biology, TCHRF, 3333 Burnet Ave., Cincinnati, OH 45229-3039.

Received 18 May 1998; accepted in final form 11 November 1998.

## REFERENCES

1. **Ballard, P. L., R. Ertsey, L. W. Gonzales, and J. Gonzales.** Transcriptional regulation of human pulmonary surfactant proteins SP-B and SP-C by glucocorticoids. *Am. J. Respir. Cell Mol. Biol.* 14: 599–607, 1996.
2. **Beers, M. F., H. Shuman, H. G. Liley, J. Floros, L. W. Gonzales, N. Yue, and P. L. Ballard.** Surfactant protein B in human fetal lung: developmental and glucocorticoid regulation. *Pediatr. Res.* 38: 668–675, 1995.
3. **Bogue, C. W., H. C. Jacobs, D. W. Dynia, C. M. Wilson, and I. Gross.** Retinoic acid increases surfactant protein mRNA in fetal rat lung in culture. *Am. J. Physiol.* 271 (*Lung Cell. Mol. Physiol.* 15): L862–L868, 1996.
4. **Bohinski, R., R. Di Lauro, and J. A. Whitsett.** The lung-specific surfactant protein B gene promoter is a target for thyroid transcription factor 1 and hepatocyte nuclear factor 3, indicating common factors for organ-specific gene expression along the foregut axis. *Mol. Cell. Biol.* 14: 5671–5681, 1994.
5. **Bourguet, W., M. Ruff, P. Chambon, H. Cronemeyer, and D. Moras.** Crystal structure of the ligand-binding domain of the human nuclear receptor RXR-alpha. *Nature* 375: 377–382, 1995.
6. **Cardoso, W. V., M. C. Williams, S. A. Mitsialis, M. Joyce-Brady, A. K. Rishi, and J. J. Brady.** Retinoic acid induces changes in the pattern of airway branching and alters epithelial cell differentiation in the developing lung in vivo. *Am. J. Respir. Cell Mol. Biol.* 12: 464–467, 1995.
7. **Chen, H. W., R. J. Lin, R. L. Schiltz, D. Chakravarti, A. Nash, L. Nagy, M. L. Privalsky, Y. Nakatani, and R. M. Evans.** Nuclear receptor coactivator ACTR is a novel histone acetyltransferase and forms a multimeric activation complex with P/CAF and CBP/p300. *Cell* 90: 569–580, 1997.
8. **Clark, J. C., S. E. Wert, C. J. Bachurski, M. T. Stahlman, B. R. Stripp, T. E. Weaver, and J. A. Whitsett.** Targeted disruption of the surfactant protein B gene disrupts surfactant homeostasis, causing respiratory failure in newborn mice. *Proc. Natl. Acad. Sci. USA* 92: 7794–7798, 1995.
9. **Damm, K., R. A. Heyman, K. Umesono, and R. M. Evans.** Functional inhibition of retinoic acid response by dominant negative retinoic acid receptor mutants. *Proc. Natl. Acad. Sci. USA* 90: 2989–2993, 1993.
10. **Dolle, P., E. Ruberte, P. Leroy, G. Morriss-Kay, and P. Chambon.** Retinoic acid receptors and cellular retinoid binding proteins I. A systematic study of their differential pattern of transcription during mouse organogenesis. *Development* 110: 1133–1151, 1990.
11. **Durand, B., M. Saunders, C. Gaudon, B. Roy, R. Losson, and P. Chambon.** Activation function 2 (AF-2) of retinoic acid receptor and 9-cis retinoic acid receptor: presence of a conserved autonomous constitutive activating domain and influence of the nature of the response element on AF-2 activity. *EMBO J.* 13: 5370–5382, 1994.
12. **George, T. N., O. L. Miakotina, K. L. Goss, and J. M. Snyder.** Mechanism of all *trans*-retinoic acid and glucocorticoid regulation of surfactant protein mRNA. *Am. J. Physiol.* 274 (*Lung Cell. Mol. Physiol.* 18): L560–L566, 1998.
13. **George, T. N., and J. M. Snyder.** Regulation of surfactant protein gene expression by retinoic acid metabolites. *Pediatr. Res.* 41: 693–701, 1997.
14. **Ghaffari, M., X. Zeng, J. A. Whitsett, and C. Yan.** Nuclear localization domain of thyroid transcription factor-1 in respiratory epithelial cells. *Biochem. J.* 328: 757–761, 1997.
15. **Hong, H., K. Kohli, A. Trivedi, D. L. Johnson, and M. R. Stallcup.** GRIP1, a novel mouse protein that serves as a transcriptional coactivator in yeast for the hormone binding domains of steroid receptors. *Proc. Natl. Acad. Sci. USA* 93: 4948–4952, 1996.
16. **Hui, L., P. J. Gomes, and J. D. Chen.** RAC3, a steroid/nuclear receptor-associated coactivator that is related to SRC-1 and TIF2. *Proc. Natl. Acad. Sci. USA* 94: 8479–8484, 1997.
17. **Imakado, S., J. R. Bickenbach, D. S. Bundman, J. A. Rothnagel, P. S. Attar, X. J. Wang, V. R. Walczak, S. Wisniewski, J. Pote, J. S. Gordon, R. A. Heyman, R. M. Evans, and D. R. Roop.** Targeting expression of a dominant-negative retinoic acid receptor mutant in the epidermis of transgenic mice results in loss of barrier function. *Genes Dev.* 9: 317–329, 1995.
18. **Kamei, Y., L. Xu, T. Heinzel, J. Torchia, R. Kurokawa, B. Gloss, S. C. Lin, R. A. Heyman, D. W. Rose, C. K. Glass, and M. G. Rosenfeld.** A CBP integrator complex mediates transcriptional activation and AP-1 inhibition by nuclear receptors. *Cell* 85: 403–414, 1996.

19. **Kastner, P., M. Mark, and P. Chambon.** Nonsteroid nuclear receptors: what are genetic studies telling us about their role in real life? *Cell* 83: 859–869, 1995.
20. **Kumar, A. S., V. C. Venkatesh, B. C. Planer, S. I. Feinstein, and P. L. Ballard.** Phorbol ester down-regulation of lung surfactant protein B gene expression by cytoplasmic trapping of thyroid transcription factor-1 and hepatocyte nuclear factor 3. *J. Biol. Chem.* 272: 20764–20773, 1997.
21. **Mangelsdorf, D. J., and R. M. Evans.** The RXR heterodimers and orphan receptors. *Cell* 83: 841–850, 1995.
22. **Mangelsdorf, D. J., C. Thummel, M. Beato, P. Herrlich, G. Schutz, K. Umesono, B. Blumberg, P. Kastner, M. Mark, P. Chambon, and R. M. Evans.** The nuclear receptor superfamily: the second decade. *Cell* 83: 835–839, 1995.
23. **Massaro, G. D., and D. Massaro.** Postnatal treatment with retinoic acid increases the number of pulmonary alveoli in rats. *Am. J. Physiol.* 270 (*Lung Cell. Mol. Physiol.* 14): L305–L310, 1996.
24. **Massaro, G. D., and D. Massaro.** Retinoic acid treatment abrogates elastase-induced pulmonary emphysema in rats. *Nat. Med.* 3: 675–677, 1997.
25. **Masuyama, H., Y. Hiramatsu., and T. Kudo.** Effect of retinoids on fetal lung development in the rat. *Biol. Neonate* 67: 264–273, 1995.
26. **Mendelsohn, C., D. Lohnes, D. Decimo, T. Lufkin, M. LeMeur, and P. Chambon.** Function of the retinoic acid receptors (RARs) during development (II). Multiple abnormalities at various stages of organogenesis in RAR double mutants. *Development* 120: 2749–2771, 1994.
27. **Mengus, G., M. May, L. Carre, P. Chambon, and I. Davidson.** Human $TAF_{II}135$ potentiates transcriptional activation by the AF-2s of the retinoic acid, vitamin D3, and thyroid hormone receptors in mammalian cells. *Genes Dev.* 11: 1381–1395, 1997.
28. **Metzler, M., and J. M. Snyder.** Retinoic acid differentially regulates expression of surfactant-associated proteins in human fetal lung. *Endocrinology* 133: 1990–1998, 1993.
29. **Nogee, L. M., D. E. DeMello, L. P. Dehner, and H. R. Colten.** Brief report: deficiency of pulmonary surfactant protein B in congenital alveolar proteinosis. *N. Engl. J. Med.* 328: 406–410, 1993.
30. **Nogee, L. M., G. Garnier, H. C. Dietz, L. Singer, A. M. Murphy, D. E. DeMello, and H. R. Colter.** A mutation in the surfactant protein B gene responsible for fatal neonatal respiratory disease in multiple kindreds. *J. Clin. Invest.* 93: 1860–1863, 1994.
31. **Onate, S. A., S. Y. Tsai, M. J. Tsai, and B. W. O'Malley.** Sequence and characterization of a coactivator for the steroid hormone receptor superfamily. *Science* 270: 1354–1357, 1995.
32. **O'Reilly, M. A., J. C. Clark, and J. A. Whitsett.** Glucocorticoids enhance pulmonary surfactant protein B gene transcription. *Am. J. Physiol.* 260 (*Lung Cell. Mol. Physiol.* 4): L37–L43, 1991.
33. **Pryhuber, G. S., S. L. Church, T. Kroft, A. Panchal, and J. A. Whitsett.** 3′-Untranslated region of SP-B mRNA mediates inhibitory effects of TPA and TNF-α on SP-B expression. *Am. J. Physiol.* 267 (*Lung Cell. Mol. Physiol.* 11): L16–L24, 1994.
34. **Pryhuber, G. S., M. A. O'Reilly, J. C. Clark, W. M. Hull, I. Fink, and J. A. Whitsett.** Phorbol ester inhibits surfactant protein SP-A and SP-B expression. *J. Biol. Chem.* 265: 20822–20828, 1990.
35. **Salbert, G., A. Fanjul, F. J. Piedrafita, X. P. Lu, S. J. Kim, P. Tran, and M. Pfahl.** Retinoic acid receptors and retinoid X receptor α regulate the transforming growth factor-β1 promoter by antagonizing AP-1 activity. *Mol. Endocrinol.* 10: 1347–1356, 1993.
36. **Shenai, J. P., F. Chytil, A. Jhaveri, and M. T. Stahlman.** Plasma vitamin A and retinol-binding protein in premature and term neonates. *J. Pediatr.* 99: 302–305, 1981.
37. **Shenai, J. P., F. Chytil, and M. T. Stahlman.** Vitamin A status of neonates with bronchopulmonary dysplasia. *Pediatr. Rev.* 19: 185–188, 1985.
38. **Shenai, J. P., K. A. Kennedy, F. Chytil, and M. T. Stahlman.** Clinical trial of vitamin A supplementation in infants susceptible to bronchopulmonary dysplasia. *J. Pediatr.* 111: 269–277, 1987.
39. **Torchia, J., D. W. Rose, J. Inostroza, Y. Kamei, S. Westin, C. K. Glass, and M. G. Rosenfeld.** The transcriptional coactivator p/CIP binds CBP and mediates nuclear-receptor function. *Nature* 387: 677–684, 1997.
40. **Venkatesh, V. C., D. M. Iannuzzi, R. Ertsey, and P. L. Ballard.** Differential glucocorticoid regulation of the pulmonary hydrophobic surfactant proteins SP-B and SP-C. *Am. J. Respir. Cell Mol. Biol.* 8: 222–228, 1993.
41. **Venkatesh, V. C., B. C. Planer, M. Schwartz, J. N. Vanderbilt, R. T. White, and P. L. Ballard.** Characterization of the promoter of human pulmonary surfactant protein B gene. *Am. J. Physiol.* 268 (*Lung Cell. Mol. Physiol.* 12): L674–L682, 1995.
42. **Voegel, J. J., M. J. S. Heine, C. Zechel, P. Chambon, and H. Gronemeyer.** TIF2, a 160 kDa transcriptional mediator for the ligand-dependent activation function AF-2 of nuclear receptors. *EMBO J.* 15: 3667–3675, 1996.
43. **Whitsett, J. A., L. M. Nogee, T. E. Weaver, and A. D. Horowitz.** Human surfactant protein B: structure, function, regulation and genetic disease. *Physiol. Rev.* 75: 749–757, 1995.
44. **Wispe, J. R., J. C. Clark, B. B. Warner, D. Fajardo, W. E. Hull, R. B. Holtzman, and J. A. Whitsett.** Tumor necrosis factor-alpha inhibits expression of pulmonary surfactant protein. *J. Clin. Invest.* 86: 1954–1960, 1990.
45. **Yan, C., M. Ghaffari, J. A. Whitsett, X. Zeng, Z. Sever, and S. Lin.** Retinoic acid receptor activation of SP-B gene transcription in respiratory epithelial cells. *Am. J. Physiol.* 275 (*Lung Cell. Mol. Physiol.* 19): L239–L246, 1998.
46. **Yan, C., Z. Sever, and J. A. Whitsett.** Upstream enhancer activity in the human surfactant protein B gene is mediated by thyroid transcription factor 1. *J. Biol. Chem.* 270: 24852–24857, 1995.
47. **Yan, C., and I. Tamm.** Molecular cloning and characterization of additional factors that bind to the interferon-α/β response element B of the murine (2′-5′)oligoadenylate synthetase ME-12 gene. *Proc. Natl. Acad. Sci. USA* 89: 8859–8863, 1992.
48. **Yan, C., and J. A. Whitsett.** Protein kinase A activation of the surfactant protein B gene is mediated by phosphorylation of thyroid transcription factor 1. *J. Biol. Chem.* 272: 17327–17332, 1997.

# Inhibition of hSP-B promoter in respiratory epithelial cells by a dominant negative retinoic acid receptor

Manely Ghaffari, Jeffrey A. Whitsett and Cong Yan

*AJP - Lung* 276:398-404, 1999.



***AJP - Lung Cellular and Molecular Physiology*** publishes original research covering the broad scope of molecular, cellular, and integrative aspects of normal and abnormal function of cells and components of the respiratory system. It is published 12 times a year (monthly) by the American Physiological Society, 9650 Rockville Pike, Bethesda MD 20814-3991.  ISSN: 1040-0605, ESSN: 1522-1504. Visit our website at http://www.the-aps.org/.

# This Page is Inserted by IFW Indexing and Scanning Operations and is not part of the Official Record

## BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☑ BLACK BORDERS
- ☐ IMAGE CUT OFF AT TOP, BOTTOM OR SIDES
- ☐ FADED TEXT OR DRAWING
- ☐ BLURRED OR ILLEGIBLE TEXT OR DRAWING
- ☐ SKEWED/SLANTED IMAGES
- ☐ COLOR OR BLACK AND WHITE PHOTOGRAPHS
- ☐ GRAY SCALE DOCUMENTS
- ☐ LINES OR MARKS ON ORIGINAL DOCUMENT
- ☐ REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY
- ☐ OTHER: _____

**IMAGES ARE BEST AVAILABLE COPY.**
**As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.**